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CARBOHYDRATE METABOLISM AND INSULIN

BY

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EDITOR'S PREFACE.

In no science is the advance at any one time general. Some sections of the line are pushed forward while other parts may remain for years with little movement, until in their turn they are enabled to progress in consequence of the support afforded by the advance of the adjacent sections. The increasing number of series of monographs in different sciences is a recognition of this fact, as well as of the concentration of interest which characterizes this age of specialization.

In the present series it is intended to set out the progress of physiology in those chapters in which the forward movement is the most pronounced. Each monograph will contain an account of our knowledge of some particular branch of physiology, written by one who has himself contributed in greater or less degree to the attainment of our present position. It is hoped that by securing the help of men who are actively engaged in the advance of the subject the outlook of each monograph will be forwards rather than backwards. An exhaustive account of previous writings on the subject concerned is not aimed at, but rather an appreciation of what is worth retaining in past work, so far as this is suggestive of the paths along which future research may be fruitful of results. The more valuable the monographs in inspiring the work of others, the greater will be the success of the series.

ERNEST H. STARLING.

October, 1915.

AUTHOR'S PREFACE.

THE main purpose of the present monograph is to give a comprehensive review of the advances which have been made in our knowledge of the metabolism of the carbohydrates during recent years, and more especially since insulin became available. This is preceded by an account of the researches which led up to the isolation of this hormone and a review of the evidence that it is derived from the Isles of Langerhans of the pancreas. The nature of the diabetic condition which supervenes upon withdrawal of insulin from the body is also discussed.

It has proved to be no easy task to study carefully, and to place in what appear to be their true relationships, the results contained in the numerous papers which have been published, and if undue weight may appear to be given to the work which has gone on under my own supervision it is hoped that this will be pardoned on the ground that otherwise it would have been impossible for me to prepare the monograph. I should like to dedicate the volume to my collaborators as a token of my appreciation and gratitude for the generous co-operation which they have shown and without which the work from my laboratory could not have been accomplished. I earnestly hope that they will consider that I have carried out my task as recorder sufficiently well to make this dedication acceptable to them.

J. J. R. MACLEOD.

TORONTO,
January, 1926.

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CHAPTER I.

THE STRUCTURE OF THE ISLETS OF LANGERHANS.

Introductory.—No useful purpose would be served by reviewing in detail the very extensive literature pertaining to the structure of the Isles of Langerhans. This has already been done by such authors as Laguesse, Diamare, Schafer, Allen, Lombroso, Warthin, Biedel, and Swale Vincent, and we will refer to the earlier investigations only in so far as is necessary for the general development of the subject. Our problem is to collect the evidence, from whatever source, which proves that the control exercised by the pancreas over the metabolism of the carbohydrates depends on the fact that this gland produces an internal secretion derived from the Isles of Langerhans. It is a tribute to anatomical investigation that this theory, now so amply proved by physiological and clinical observation, should have sprung from painstaking studies in the comparative anatomy and histogenesis of the pancreas.

Langerhans, who first described the isles in 1869, did not venture to express any definite opinion as to their physiological significance. He remarked on their close relationship to nerves (and to nerve ganglia), although he could discern no actual connections between the two. Nor did those who continued his studies make any definite hypothesis regarding function. Indeed many, such as Lea and Kühne (1882), Sokoloff (1883), Krause (1884), and Ellenberger (1887) [1] described the islets as collections of lymphoid cells, a view which was corrected, partly by the more careful histological investigations of Laguesse (1893), Harris and Gow (1894), Diamare (1895), and E. S. Schafer (1895), and partly by the observation of Gentes (1901) that the islets do not, like typical lymphoid structures elsewhere in the body, become hypertrophied in leucæmia. Although they came to be recognised as composed of epithelial cells, this did not serve to suggest to more than a few that the islets have a function essentially different from that of the cells composing the acini. Some there were who thought that they might be the source of certain

[1] References to the papers of these workers will be found in the monographs of Laguesse, Allen, Biedel, and Schafer.

of the ferments contained in the external secretion of the pancreas, such as the diastatic enzyme (Harris and Gow, 1894 ; Giannelli and Giacomini, 1896) or, if not of a ferment, at least of a co-ferment which formed trypsin, by uniting with another co-ferment transported to the islets from the spleen (Sajous, 1904). The absence of any patent duct to the islets, their relative persistence after destruction of the acini as a result of ligation of the secreting ducts of the gland, and the failure to demonstrate any digestive action in extracts made from their homologues, the principal islets of the bony fishes (p. 27), may be cited as evidence against the view that the isles of Langerhans have any digestive secretory function.

While admitting that the islets do not contribute anything to the digestive secretion of the pancreas, several observers, particularly Lewaschew (1886), Tschassownikow (1900), and Mankowski (1900-1902), thought them to be acini that had undergone some change, probably because of exhaustion; others pointed out, however, that the abundant supply of blood-vessels and nerves to the islets and their persistence after ligation of the pancreatic ducts were irreconcilable with such a view. Two well-known authorities, Giannelli (1899-1902) and Oppel (1900), as an outcome of studies in the embryology and comparative anatomy of the islets, concluded that they are vestigial remnants of a tissue which, in primitive types of animal and in the early stages of development of higher ones, possess important secretory functions. According to Giannelli, there occur in the pancreas of lower vertebrates structures having a certain secretory function, but as the animal scale is ascended, the tubules producing the secretion gradually loose their lumina and become solid cords of epithelial cells, which exist as the isles of Langerhans of the mammalian pancreas. Oppel, impressed with the compact form which the islets assume in the Elasmobranchi (p. 29), suggested that these might represent a primitive pancreas (pre-pancreas), inherited from pre-vertebral ancestors, which had lost its digestive function in favour of a new one, mediated through the production of an internal secretion. In higher types, according to this view, the islet tissue, thus derived, becomes more and more replaced by the development of new acinar tissue, although both are derived from the same embryological "anlage." This implies that the islet cells in the mammalian pancreas are more rudimentary than the acinar, a view which, as Laguesse points out, is entirely contrary to the facts. The absence of the zymogen granules does not, as is supposed by Oppel, indicate reversion to a primitive type, but rather a higher specialisation, as an outcome of which, there appear the α and β granules, to which we will refer immediately. Moreover, the total mass of islet tissue in the pancreas of man is very large and the blood supply of the islets is well developed, facts which scarcely lend support to the view that it is a vestigial structure.

The first definitely to state that the islets are endocrine organs producing an internal secretion, were Laguesse (1893) and Dia-

mare (1895), a view which was accepted by E. Sharpey Schafer (1893), who subsequently suggested (1916) that the internal secretion should be named "insuline". But Laguesse did not consider that the cells of the islets are entirely independent of those of the acini, but rather that there is a certain balance between the two kinds of cells. To quote R. R. Bensley, "he (Laguesse) thought . . . that the internal and external secretory functions of the pancreas were possessed in like measure by both acinous and islet cells, and that the formation of islets was a sort of reversal of polarity of the cell for the purposes of internal secretion." Basing his argument on the presence of intermediate types of cell, "he supposed that there was some sort of physiological mechanism of control which determined, from moment to moment, the relative amounts of the two tissues which were needed for the external and internal secretory purposes" (Harvey lecture). In support of these views it was stated: (1) That cells differing in their microchemical reactions from those both of the islets and the acini could be demonstrated in the pancreas; (2) that these supposedly intermediate types varied in numbers under different physiological conditions; and (3) that the cells of the islets could often be seen to be in apparent continuity with those of the acini. Strong support seemed also to be afforded by the investigations of Dale and Vincent and Thompson, if, indeed, as Bensley points out, their conclusions did not go so far as almost to question the reality of the islets, and so to revive the theory of Lewaschew, that these structures are really produced during the activity of the pancreas by transformation from acini, into which they again revert when the gland is rested. The observations of these workers, however, were based, not on the cytological characteristics of the cells, but on the absence of zymogen granules and basophile substance (p. 11), that is, on negative rather than positive properties, and they fail to be sustained by the more recent investigations of Lane, Bensley, Homans, and others, in which, by modern staining methods, it has been found that neither after starvation nor after exhaustion of the acini, as a result of repeated injections of secretin, are the numbers of islet cells increased over the normal.

The great majority of anatomists have leaned to the view, first clearly expressed by Diamare and endorsed by Rennie and

Schafer, that the islets are structures distinct and apart from the rest of the pancreas, with the specific function of producing an internal secretion. According to this view, the islets bear to the acini much the same relationship as the parathyroid glands bear to the thyroid, or the cortex of the suprarenal gland bears to the medulla. Notwithstanding they are anatomically associated, the physiological functions of the two kinds of cells in each pair of glands are independent. Although Diamare's view is now almost universally accepted by anatomical, as well as by experimental workers, his statement that the islets are entirely detached from the duct system of the gland is not supported by the more recent work, especially that of Bensley, in which, by methods of vital staining, definite connections between islets and ducts have been demonstrated to exist. Diamare clearly states that the islets are buds of the pancreatic system (i.e. derived from the same embryological anlage), but he maintains that they become detached from this, surrounded often by a capsule and related only to the blood-vessels.

Certain anatomists, including Renaut, impressed with the fact that a lumen can be seen between the rows of cells of the islets in certain of the lower vertebrates, such as the Ophidia, have concluded that the cells may secrete both externally and internally. Such a view is untenable in the case of the mammals although, if we accept Laguesse's teaching, that islet cells may be derived from acinar, there must be a stage at which the latter are secreting both internally and externally.

Cytological Characteristics.—The universal presence of islet cells throughout the vertebrates, their relative abundance in the fœtal pancreas, and their close association with blood-vessels supply sufficient evidence of their physiological importance. That their function is independent of that of the acinar cells is strongly indicated by careful studies of their cytological characteristics. Many of the earlier investigations bearing on this problem were done with material too imperfectly fixed and stained for accurate study, and while not losing sight of the important contributions, particularly of Laguesse and Diamare, we will start with those of Bensley and Lane, to whom many of the recent improvements in technique are due.

Using tissue that was removed from animals immediately after death, so as to avoid *post mortem* changes, Lane placed pieces of it in various fixing solutions (i.e. made with either

alcohol, or water, or acid), in order to see whether the granules, both of the acinar and the islet cells, would be differently affected. He then stained the fixed preparations with neutral gentian, to bring out differences in the micro-chemical properties of the granules. Briefly, he found that the zymogen granules of the acinar cells do not stain after fixation in alcohol (70 per cent.) alone, but do so after fixation in solutions containing potassium bicromate and mercuric chloride (chrome sublimate solution). In the islet cells two distinct types of granules could be differentiated; in sections fixed by alcohol and stained with neutral gentian large numbers of the islet cells contained no visible granules, while a smaller number were packed with them; whereas in similarly stained sections after fixation in chrome sublimate solutions, the majority of the islet cells contained deeply stained granules, and those which had contained them in the alcohol-fixed sections were now empty. Lane designated the relatively numerous cells with granules which stained after chrome sublimate fixation as β-cells, and the less numerous ones with granules which stained after alcohol fixation as α-cells. It has also been claimed that the α-cells in the mammalian pancreas are commonly distributed at the periphery of the islets, that is, nearest to the acini, which accounts for their having been considered by Laguesse, Ssobolew, Tschassownikow as intermediate, or transition forms. In the pancreas of Raja, the alpha cells are definitely situated at the edge of the islets.

The procedure necessary to distinguish the α- and β-cells has been considerably simplified by using methods depending on differential staining alone. For this purpose, the fixing solution must preserve all kinds of granules, and the stains must be capable of tinting each of these characteristically.[1] A solution containing osmic acid, potassium bichromate, and a trace of acetic acid, is recommended by Bensley for this purpose, its poor penetrating properties being overcome by using small, thin pieces of tissue, freed as much as possible from fat. As a differential stain, one containing aniline acid fuchsin and methyl green may be used, the α-cells being coloured red, and the β-cells green. In our experience the staining methods described by Martin and by Bowie are the most useful with fresh tissue (p. 16), although various other combinations of stains may be used, provided the tissue is properly fixed—for example eosin and methylene blue, as used by F. M. Allen.

[1] With other fixatives and stains the differentiation of α- and β-cells can be made according to the following scheme drawn up by D. J. Bowie (see accompanying table).

TABLE I. (Bowie).

Fixative.	Neutral Gentian.	Acid Azo-Fuchsin Basic Ethyl Violet.	Anilin Acid Fuchsin Methyl Green.	Eosin Methylene Blue.	Ethyl Violet Biebrich Scarlet.	Ehrlich's Hematoxylin and Eosin.
70 per cent. alcohol	α-Violet β-Yellow					
Alcohol chrome sublimate	α-Violet β-Yellow	α-Violet β-Red		α-Blue β-Red		
Bensley's acetic osmic bichromate			α-Deep red β-Green γ-Clear			
Zenker's (2 per cent. acetic acid)	α-Violet β-Yellow	α-Violet β-Red (brick) γ-Red (light)	α-Green β-Dull red γ-Bright red	α-Blue β-Brick red γ-Light red	α-Blue β-Purple γ-Light red	α-Dark red β-Medium red γ-Light red
Zenker's (without acetic acid)	α-Yellow β-Violet	α-Red β-Violet			α-Red β-Blue	
Neutral formalin, then acetic osmic bichromate			α-Dull red β-Green γ-Red			

In properly fixed and stained sections it is an easy matter to differentiate the islets from the acini by the positive characteristics of their cells, and there is practical agreement among most recent workers that cells intermediate between those of the islets and acini do not occur (Bensley, Lane, Homans, Allen, Cecil, and Bowie). The only contrary opinion has been that of Saguchi, who studied the general cytological characteristics of

the cells (mitochondria, nuclear contents, appearance of granules), rather than the tinctorial reactions of the granules, and who concluded, from studies on the frog's pancreas, that there is a very gradual transition from zymogenous to islet cell and that five different kinds of islet cell can be differentiated. The gamma cells described by Bensley in the mammalian pancreas, and observed by Bowie to be relatively plentiful in the principal islets of certain fishes, may possibly be transitional between the duct cells and the alpha and beta cells.

Not only are the cells of the islets and acini to be distinguished on account of the properties of their granules, but their other cytological characteristics are also different. *The cell of the acinus* contains zymogen granules only in its inner two-thirds, the outer third being occupied by a more or less homogeneous substance which stains with basic dyes, and is called the chromidial substance of Hertwig. Under certain conditions, fresh preparations show a faint radial striation, and in fixed specimens stained with fuchsin and methyl green the striations are seen to be due to long filaments, staining red. These are the mitochondrial filaments common to all cells, but of unusual size in those of the acini. The nuclei are large, rich in chromatin, and they contain a large oxyphile nucleolus. In appropriately stained preparations, a network of fine channels containing clear fluid may be seen in the cytoplasm near the inner pole of the nucleus. *The centroacinar cells and the cells of intralobular ducts* are readily distinguishable by the absence of zymogen granules, the small, irregularly distributed mitochondria, and the absence of chromidial substance.

The islet cells, in their general cytological characteristics, are more like those of the ducts and the centroacinar cells than those of the acini; they have no chromidial substance, the mitochondria are scattered as minute filaments throughout the cytoplasm and no large oxyphile nucleolus is visible, though small ones are often present. A study of these characters, along with recognition of the granules peculiar to each kind of cell, is necessary before conclusions as to the presence of transition forms between those of acini and islets can be drawn; and when this is done, as Bensley has pointed out, there remains no evidence of transition forms. Sometimes, as in the pancreas of guinea-pigs fed for some time on a diet of oats and hay, a peculiar change may

occur in the outer third of the acinar cells, the chromidial substance becoming replaced by granules (Mankowski granules) which are somewhat larger than those of the islet cells, and are insoluble both in alcohol and water. These may increase in numbers until they replace the zymogen granules. The mitochondrial filaments also break up and dissolve in the protoplasm, which indicates that a pathological change has occurred. This change proceeds independently of any change in the islets, and these may be seen, in suitably stained sections, alongside acini containing the degenerated cells.

It is unfortunate that the methods of fixing and staining necessary to bring out these cytological characteristics of the islet and acinar cells can seldom be used successfully with the pancreas of patients who have died of diabetes. Could this material be obtained immediately after death it is unlikely that the transition forms of cells which many pathologists have described would be seen.

Enumeration of the Islets.—Even when these requirements of technique are fulfilled, the sections are only suitable for purposes of differentiation of the cells, and they cannot properly be used as the basis for an estimate of the relative numbers of acini and islets, and only occasionally for a study of the possible relationship of the islets to the pancreatic ducts and blood-vessels. To study these relationships it is necessary that vitally stained preparations be used, and the technique required to do this we again owe largely to R. R. Bensley. This worker, using the guinea-pig, determined the relative number and the general distribution of the islets, by injecting either janus green (1-15,000 in physiological salt solution) or neutral red into the aorta, immediately after the death of the animal.

With janus green the entire pancreas stains at first a deep blue, but if the preparation be covered up so as to exclude air, reduction of the dye occurs, proceeding more rapidly in the acinar tissue than in the islets, so that the former gradually becomes of a red colour, whilst the islets are still a deep blue. The colours may be fixed at this stage by injection of a solution of ammonium molybdate into the duct, and if pieces of the pancreas be now removed and quickly washed in ice-cold water and absolute alcohol, they may be clarified by toluol and pressed out under a cover slip. Actual counts can then be made of the number of islets (see Fig. 1). The neutral red preparations made in the same way have the disadvantage that they cannot be rendered permanent,

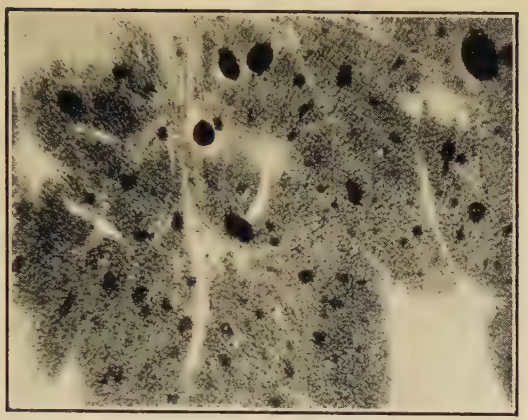

FIG. 1.—Photomicrograph of preparation of the pancreas of the guinea pig, made by injection of neutral red into the blood-vessels, × 38. (From R. R. Bensley, "Amer. Jour. Anat.," 1911, xii, 297.)

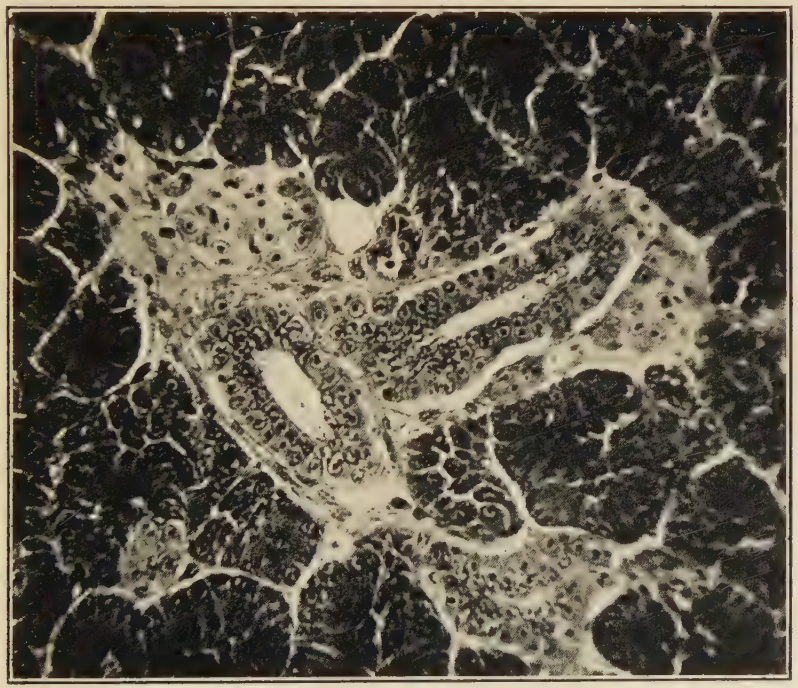

FIG. 2.—Section of pancreas of skate, × 320. Note the continuity of islet tissue with the outer layer of cells of the duct. Iron hæmatoxylin and congo red. (From Slater Jackson, "Jour. Metabolic Research," 1922, II.)

but Bensley appears to prefer them when the object is to make actual counts of the islets.

By these methods, " the enormous quantity of the islet tissue becomes at once apparent, and the inaccuracy of estimates made on the examination of sections can be perceived and computed."

In the pancreas of guinea-pigs, varying in weight between 300 and 600 gms., the average number of islets per cubic millimetre of pancreas was found to be 22·28, which is about twenty times as many as that given by Laguesse and Dewitt, based on counts made with sections. This discrepancy is readily explained by the fact that the injection methods reveal clearly islets, or isolated islet cells, that are entirely missed in sections stained by the usual technique. No indefinite transition forms are seen ; the islets, on the contrary, stand out sharp and clear as histological entities. In the entire pancreas of the guinea pig as many as 56,000 islets were counted, indicating that this endocrine tissue, instead of being rather scant, as it has usually been thought to be, is relatively abundant.

Comparison of the numbers of islets under different conditions showed that there is great variability, not only in the pancreases of different animals, but also in different portions of the pancreas of the same one, although these portions might be closely adjacent. It is scarcely necessary to point out that these results indicate the futility of attempting to form an estimate of the effect of physiological or pathological conditions on the numbers of islets by counting those seen in similar areas in a few sections of the gland. Even when pains are taken to use carefully ruled slides for counting, and numerous sections are selected from each portion of the pancreas, serious errors are bound to enter in. The closest approach to quantitative accuracy, preceding the work of Bensley and his collaborators, is probably that of Heiberg, in which outline drawings of the islets in carefully selected sections were made on thick paper, which was then cut out and the islet patterns weighed. Based on such counts important conclusions have been drawn, especially concerning the transformation of acini into islets, or *vice versa*, but it has been impossible to confirm this conclusion by use of the newer methods.

The results obtained by Bensley's and Heiberg's methods are in agreement in showing that the islets are more numerous in the tail (splenic end) than in the body or head of the gland.

Bensley and his co-workers made counts in forty-six guinea-pigs on the basis of the number of islets in 1 mg. of pancreas, the results being obtained by enumerating the islets in small pieces, removed from the three regions of the pancreas, and then weighing the pieces. The splenic portion gave the highest average in 39 cases, and the second highest in 7; the body was highest in 7, second in 29, and third in 10; the duodenal portion was highest in none, second in 10, and third in 36. As typical figures, the following may be given :—

Weight of guinea-pig—647 gms.	
Number of islets per mg. pancreas—splenic portion . .	31·3
,, ,, ,, —body portion . .	22·4
,, ,, ,, —duodenal portion .	17·0

The islets are much more numerous in the pancreas of young animals. Thus, in a guinea-pig weighing 74 gms. the number of islets, per milligram of pancreas, were :—

Splenic portion	190
Body portion	161
Duodenal portion	156

The differences are probably even greater than those indicated by the figures, since it is difficult to count the islets in the pancreas of young animals, because they are so close together.

Without going further into detail with regard to the relative distribution of islets, it may be stated that this is not constant even with animals of the same ages and weights, and that it varies greatly, not only in different portions of the same pancreas, but also in closely adjacent portions. Clearly, therefore, no estimate of the total number of islets can be dependable; they must actually be counted in the entire gland after its injection with janus green or neutral red.

The most important application of these methods has been in refuting the conclusions, which at one time were drawn by H. H. Dale and by Swale Vincent and Thompson, concerning the effect produced on the relative number of islets by various physiological conditions, especially inanition and over-stimulation of the external secretory (acinar) cells by repeated injections of secretin. It was thought that a considerable increase occurred in the number of islets under these conditions, without any cell division but merely because of exhaustion of the cells of the acini. The importance of this conclusion, as Bensley points out, is not alone that it would degrade the islets, from the position of independent structures having a specific endocrine function

of their own, to one of merely exhausted acini, but also, that it would have a far-reaching significance in showing that dissimilarity in structure of different parts of a compound organ cannot be depended upon as evidence of possible differences in function. The apparent transmutability of acini and islets seemed to question the reality of the islets, and Bensley undertook to confirm or refute the evidence by actual counts.

The results with secretin were clearly contrary to those of Dale. In the guinea-pig, for example, eight to ten hours after the subcutaneous injection of secretin, the acinar cells of the pancreas were found to be very thoroughly exhausted of zymogen granules and those of the islets to be deeply and uniformally stained by neutral red, without any evidence of transition forms of cells. The total numbers of islets fell within the limits found for the pancreas of normal, uninjected animals, previously bred and reared under uniform laboratory conditions. Similar results were obtained with the toad, an animal which responds well to secretin. After being kept under the influence of secretin for from four to seven days, neither was the number of islets greater than in control animals, nor could transition forms of cells be detected.

The results were of the same nature after inanition. In this case, besides Dale, Statkewitsch (1894), Vincent and Thompson (1907), and Laguesse (1910) had described an increase of islet tissue, the explanation given for the result being that inanition has an effect on the secretory granules (of the acini) similar to that of over-stimulation. But even if this were the case, and there is no obvious reason why it should be so, it does not necessarily mean that the exhausted cells of the acini, *ipso facto*, become islet cells. The most careful work of this character was done in 1911 by Laguesse on pigeons, the number of islets per square mm. being, in controls, 4·5 ; in birds starved from five to ten days, 7·8 ; and in those first of all starved and then fed, 4·3. This author saw in these results evidence to favour his view that islets under certain conditions might become produced at the expense of acini, although he believed the two structures to have independent functions. Neither in the guinea-pig nor in the dog could evidence be obtained, by the neutral red or janus green methods of staining, of a relative increase in islet tissue following inanition.

Quite apart from their importance in refuting the evidence upon which the various theories of interrelationship between acini and islets has depended, the results of these investigations have set a standard for future work, and they make it clear that the islets are just as independent of the acini as are the parathyroid glands of the thyroid vesicles. This conclusion is further sustained by a study of the blood supply of the pancreas, this

being peculiarly rich to the islets. To reveal them the blood-vessels are injected with carmine gelatine, and this can be done after vital staining with janus green, followed by appropriate reduction of the stain. The walls of the arterioles in such preparations take the blue colour of the janus green, the capillaries are purple and the veins bright red, and it can be seen that at least one arteriole runs to each islet, often several to the larger ones, and that even isolated islet cells stand in close relationship to a dilated capillary loop. There are no sinusoids. There can be no doubt of the fact, first pointed out by Kühne and Lea, that the islets, like other ductless glands, are particularly well supplied with blood-vessels. Not even the adherents of the mutation hypothesis deny this high vascularity, but they attempt to explain it as due to the shrinkage of the tissue when acini pass into islets.

Very little that is definite appears to be known with regard to the *embryology of the islets*. The earliest authoritative account is that given by Laguesse who, as a result of work done between 1894 and 1897, concluded, from studies on embryo sheep, that islands become developed from the ductule system before the acini have differentiated, but later that secondary islets arise from the acini. The primary islets degenerate, but the secondary ones become extensively vascularised and increase in size and number until a "state of balance" with the needs of the organism for internal secretion is reached. Diamare (1899-1905), on the other hand, stated that the islets arise as solid branching cords which become extensively vascularised and then separated from the ductule system. R. M. Pearce, in 1903, described the islets in the pancreas of a human fœtus of 54 mm. as "small groups of cells lying at the side of a glandular process and in direct continuity with it . . . composed of ten to fifteen cells which have round, lightly-staining nuclei and a comparatively large amount of finely granular protoplasm staining deeply with eosin." In later fœtuses the islets were found to become isolated from the ductule system and surrounded by acinar tissue so that they occupied a central position in the acini. Knester (1904), however, could still recognise connections with epithelial cords. Weichselbaum and Kyrle (1909), confirmed, in general, the description given by Pearce, but did not agree that the islets become surrounded by acinar tissue. In 6 mm. embryos of the porpoise, Helly (1906) observed differentiated cells, in solid pancreatic anlage, which he believed to be the precursors of islets. Since none of the modern methods of differential staining for the alpha and beta granules of the islet cells were used in these investigations, great difficulties were encountered in differentiating.

It is obvious that a hopeful way of approach of this problem is to study the principal islets at various stages in the development of such

fish as Myoxocephalus. With this work W. C. M. Scott is engaged and, although it is not yet completed, some facts of importance have come to light of which the following is a brief summary kindly given me by Mr. Scott: "In the youngest larva yet examined (5·5 mm.) the island which later becomes attached to the spleen is already isolated and encapsulated. Later an evagination of the pancreatic duct near the intestine occurs which, by subdivision, forms the group of islands near the bile duct. The splenic island is already isolated and encapsulated in this species (Myoxocephalus) at a time when the splenic anlage has not differentiated, and in this respect its origin varies from the before-mentioned descriptions. In the fœtus of 54 mm. in man, described by Pearce, the splenic anlage would be well developed. We believe from the studies thus presented that the islands arise from the ductule system and possibly also from the primitive differentiations of the ductules which, in their adult form, are known as acini. These islands retain their connection with the excretory tree for an indefinite period. Before the interval of differentiation becomes known some more accurate method of determining the amount of islet tissue present in the organism at any given stage of development than has been used up to the present must be at hand."

Relationship of Islets to Pancreatic Ducts.—In the simple type of pancreas seen in the Elasmobranchi (Raja) there can be no doubt of the close relationship of the islet tissue to the pancreatic ducts. A review of the earlier work of Diamare, Oppel, etc., will be found in the paper by Slater Jackson (1922), in which it is also shown (Fig. 2) that large irregularly shaped masses of islet cells are definitely arranged along the duct system. Smaller islets, shaped more or less like those in the mammalian pancreas, can also be seen, here and there, embedded among the acini (at left of section), and in no evident relationship to the ducts, but it is probable that this is merely because, in sections in one plane, processes extending from the branching islet tissue are cut across. By the use of Lane's methods, Slater Jackson has found that "both large and small islands . . . consist entirely of two types of cell, differing in size, shape, relation, number, and chromatin content," "the cytoplasm of the larger cells in sections fixed with alcohol (α-cells) being filled with minute deeply staining granules, while the smaller and more numerous (β-cells) appear clear." On the other hand, in sections fixed in non-alcoholic solutions, the β-cells exhibit granules, whilst the α-cells remain clear. A most important observation is that certain of the cells of the outer layer of duct epithelium stain exactly like α-cells. This is seen clearly in the section shown in Fig. 2, and it means, without

doubt, that these cells, at least, have their origin from the ducts. This supports the views of Helly, Pearce, etc., of the embryological origin of the islet cells from the ducts, and it conforms with the conclusion drawn by Weichselbaum and Kyrle, "that the duct epithelium possesses the power of forming islets even in adult life." These observations are of great importance in connection with the possible regeneration of islet tissue in diabetic cases, in which, by proper treatment, the islets have been relieved of all strain.

Clear though these relationships may be in the primitive pancreas of Raja, this does not necessarily imply that the islets have any duct connections in the pancreas of the mammalia; indeed, Pearce (1903) and others describe the islets as losing these connections in the course of development. On the other hand, using specimens injected through the ducts, Lewaschew (1886) and Laguesse (1893-94) describe duct connections, although no lumina could be made out. After reviewing the various opinions in this connection, Laguesse sums up by saying, that in general the ducts (canaux) do not penetrate the islets, but usually one or several fine branches run to the latter, for which they serve as pedicles. Because of shrinkage of the islets (in the process of fixing, etc.), however, these pedicles frequently become broken.

It is evident that sections are unsuitable to demonstrate these duct connections with certainty, for not only are the finer ones liable to become broken, but even if they are not, examination in one plane may fail to reveal their presence. Here, again, we owe to Bensley the development of more satisfactory methods, as a result of which we are now certain that the islets are connected with the duct system by cords of epithelial cells which are either solid, or if they possess a lumen, have this shut off before the islet is reached, by a barrier of duct cells. The ducts may be stained by injecting through the aorta with pyronin, acridin red, or methylene blue. Pyronin can be combined with janus green, neutral red, or methylene blue, giving differential staining of the ducts and islets. Thus, with pyronin and janus green, the ducts, even up to the centro acinar cells, stain deeply of a rose-red tint, and the islet cells, slate-blue. Methylene blue staining alone (intra vitam) is excellent for demonstration of the smallest ducts and centro acinar cells, the nerve fibres being also stained. The ramifications of the ducts can be best brought

FIG. 3.—Duct with branches showing the highly-branched tubules connected with the duct and with an islet. Intra vitam staining with pyronin and neutral red, × 77. (From R. R. Bensley, "Amer. Jour. Anat.," 1911, xii, 297.)

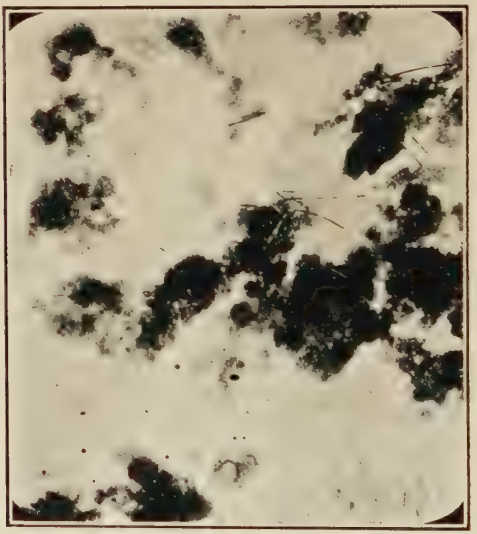

FIG. 4.—Photomicrograph of rabbit pancreas following ligation, showing regeneration of islets after 533 days. (R. R. Bensley.)

out by using " total " preparations (i.e. pieces of tissue pressed out) instead of sections, although these are necessary to demonstrate the connections of islets deeply embedded in the acinar lobules. It is important to emphasise that the entire duct system of the pancreas cannot be injected through the main duct, the reason being that the fine epithelial cords, which will be described immediately, are filled with mucus and are closed at their far ends so that no injection mass can enter them.

As a result of the use of these methods on the pancreas of the guinea-pig, Bensley has shown that a remarkable system of fine branching, tortuous epithelial tubules, or cords, extend from the larger ducts (Fig. 3). The branches often anastomose with one another, or they may become connected with islets. Smaller islets may be attached sessile on the branches, on which also bulgings, due to small mucous glands, may be observed. Acini can sometimes be seen attached to the tubules. The walls of the tubules are composed of cubical epithelial cells, the nuclei of which often show mitosis ; here and there some of them take the islet stains. Evidently " we have to do here with a tissue of a low order of differentiation, which is capable, under proper conditions, of producing by differentiation and by mitotic division islets, acini and mucous glands."

By using specimens doubly stained to show ducts and islets, Bensley divides the latter into four groups :—

1. Islets in the interstitial tissue of the pancreas quite unconnected with acini but lying near the ducts with which they are either directly connected, or connected through epithelial tubules.

2. Islets lying within the lobules unconnected with the acini, but joined to the interlobular duct system by epithelial tubules.

3. Islets lying in the lobules and continuous with the acini or with the ducts.

4. Islets of groups 1 and 2 which have lost their tubular connections. Most of the islets belong to class 3, that is, they are in intimate association with acini, as Laguesse also maintains. No connective tissue fibrils are interposed between the islet and acinar cells, the cells being in juxtaposition, but Bensley has never succeeded in discovering any transitional form of cells, such as would be required by Laguesse's hypothesis of a reciprocal interchange of the one kind with the other. The important point to remember is that all the islets of the pancreas, with few exceptions, are connected in some way with the duct system, although, as in the islets of group 3, the connection may not be visible, especially in ordinary sections of the gland.

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CHAPTER II.

STRUCTURAL CHANGES IN THE PANCREAS UNDER VARIOUS EXPERIMENTAL CONDITIONS.

A very large number of investigations have been made for the purpose of differentiating the islet from the acinar, or zymogenous tissue. It is evident that since the latter alone possesses external ducts, ligation of these would be expected to produce structural changes in the acinar cells before the insular. In the earlier experiments, in which such ligation was attempted, it was soon observed that simple ligation of the main pancreatic duct, in the dog at least, does not permanently cut off the duct system of the gland from the duodenum. This is partly because supernumerary ducts are frequent (Otto Hess, 1907), and partly because new ones may become developed under certain conditions (Allen, 1913). As was originally pointed out by Ssobolew (1902), this possible regeneration of ducts may be prevented by cutting across every possible duct connection and then stitching a fold of mesentery so that it lies between the cut ends.

The technical difficulties met with in the duct-ligation experiments have been, in large part, overcome by using grafts of the gland transplanted usually under the skin of the abdomen, so that reconnection by ducts with the duodenum cannot occur. Here also, however, a certain amount of confusion in the interpretation of the structural changes is occasioned by the considerable growth of fibrous tissue in the graft. The most recent method is that introduced by Allen, in which a great part of the pancreas is removed, the portion left retaining normal duct connection with the duodenum, so that the acinar cells, having a free pathway of external secretion, do not undergo any changes. The islet cells, on the other hand, undergo cytological changes, which are supposed to be dependent upon the strain due to their having to produce sufficient internal secretion for the needs of the body. We will consider briefly some of the most outstanding of these changes.

I. Ligation of the Ducts.—A very good review of the earlier work on this subject is given in Allen's monograph.

Among the earliest of the observations were those of D'Arnozan and Vaillard (1884) in which the pancreatic duct was ligated. Although most of the gland degenerated certain structures persisted, but it is not clear whether these were composed of acinar or islet cells. After the islets had come to be differentiated anatomically from the acini, Ssobolew studied the histological changes following ligation and cutting of the pancreatic ducts, or their injection with oil. In all the animals examined (dogs, cats, and rabbits) a considerable amount of interlobular fibrosis was found, the acini being entirely atrophied, but structures, identified as the islets, remaining apparently normal. In the process of atrophy the acinar cells are described as first of all losing their granules, thus making them appear like duct cells which, later on, often showed considerable regeneration. This regenerative proliferation of the duct cells was particularly well marked in the rabbit's pancreas. When the pancreas was left for over a year after the ligation of the ducts the interlobular fibrous tissue was found to have become replaced by fat, embedded in which were groups of cells identified as islets, since no zymogen granules could be seen in them nor any duct connections detected. At about the same time, Schulze (1900) also reported observations in guinea-pigs in which a small fragment of the pancreas was isolated from the remainder. The acini were found to disappear while the islets remained embedded in fibrous tissue.

It is mainly as an outcome of the work of these two authors that investigations of the pancreas following complete severance of the duct system has been intensively investigated in various laboratories during recent years. In 1905 Diamare observed, after oil injection of the pancreatic duct in dogs, that both the islets and acini disappeared, and in 1908 he found that complete isolation of the pancreas in frogs caused the gland to atrophy, so that in the residue only a few acini remained, although numerous large islets were still preserved. Tschassownikow (1906) also found islets to be preserved, but the rest of the gland to be degenerated, at intervals up to seventy-five days after ligation of the ducts, in rabbits.

About this time the view of Laguesse, that islets may, under certain conditions, become developed out of acinar tissue, attracted considerable attention, and various workers described transition forms of cells following duct ligation. Among these may be mentioned Marrassini (1907) who, working on rabbits, modified the previous experiments to the extent that he injected the operated animals with dextrose. In sixty days, after dividing the ducts, islets were still found present, although many had apparently disappeared. Gelle (1908) described very similar changes. After ligation of the ducts of the guinea-pig Laguesse himself found, with Gontier de La Roche, that the acini soon disappeared, whereas the islets remained, although at about the end of the second month, as a consequence of their invasion by sclerotic

tissue, many of them had undergone atrophy. In similar observations on rabbits, in which some of the animals were kept alive for between three and four years after the operation, the ducts and acini were found to have completely disappeared, the islets, however, remaining, particularly towards the splenic end. A full description of these observations will be found in Laguesse's monograph.

It would appear, therefore, that the acini degenerate rapidly and completely, whereas the islets remain more or less intact, although this view has not been endorsed by all investigators. Gradual destruction of the islets is described by Pende and by Carraro in ligation experiments on rabbits (cf. Allen), and Pratt and Spooner (1911) found that practically all pancreatic tissue disappears following severance of the ducts in dogs.

There can be no doubt that the lack of uniformity in the results has been in part due to incomplete disconnection of the pancreatic ducts from the intestine, and many authors who have reported preservation of both islet and acinar tissue do not give evidence that this source of confusion has been adequately controlled in their work. Avoiding this source of confusion, Tiberti (1909) paid particular attention to regeneration of acinar tissue, following the initial degeneration of the cells. In rabbits, during the first month or two after ligation, he found only atrophic acini, whereas after two-and-a-half months, normal ones with cells containing zymogen granules were seen, although not abundantly. After four or five months, there were some practically normal islets accompanied by others in various stages of atrophy, particularly towards the splenic end. In other experiments on dogs this author describes preservation of both islets and acini. Lombroso (1910) found, in the pancreas of the pigeon, that ligation of one lobe led to rapid atrophy of acinar cells without perceptible changes in the islets, this atrophy being, however, followed in about two months by regenerative processes. As the result of experiments in which different lobes were ligated after varying intervals, so that the acini might have regenerated in one lobe before the process of degeneration was brought on in another, this author proposed the hypothesis that the acini must furnish an internal secretion in some way connected with metabolism. He found, when those ligations were made in such a way that no normal or regenerated acinar tissue was present, that the animals died of cachexia, which was not due to diabetes, since the islets were intact. Repetition of experiments of this type on different animals, such as the dog and rabbit, led Lombroso to sound a note of warning as to the generalisation of findings obtained in experiments on one species of animal. But the chief objection to the duct-ligation investigations depends on the uncertainty that the secretory pathway has really been permanently blocked.[1]

[1] That regeneration of ducts may occur is illustrated in an observation of Clark's described by Bensley in his Harvey lecture. The duct was cut between two ligatures in a rabbit, and fifteen months later a new communication was found to

In investigations in Bensley's laboratory, by Clark, the primary result of duct ligation was found to be almost complete degeneration of acini and probably of the smallest islets which are intimately associated with them. At the end of seven days, in the guinea-pig, many cells, indistinguishable in structure from duct cells, were found, and were regarded as de-differentiated acinar cells, " because of their imbrication over the ends of the interlobular ducts." These and the duct cells which they resembled showed many mitoses, indicating probably that the regenerative processes, evident at later stages, had originated from these cells. In one month these regenerative processes were quite distinct, resulting in the formation of new islets and new acini, but at the same time the large original islets, still visible after a week, were being invaded by the growth of sclerotic tissue. The new islet tissue appeared to spring from the duct system, to which it remained attached, and it became progressively more abundant. On the other hand, the acini, although they were constantly being formed, retrograded again and became de-differentiated. " The new formed acini, as soon as they reach the point where they are able to secrete, become cystic and undergo retrograde changes." In five-and-a-half months the islet tissue was spread in branching masses along the duct system, giving a picture not at all unlike that described by Slater Jackson in the case of the primitive pancreas of the skate (see Fig. 2, p. 8). It stained with the vital stains, and its cells contained the characteristic granules. Meanwhile, the acini were seen to be fewer in number, but still distinctly present, as evidenced by their cells containing zymogen granules. How long it takes, after ligation of the duct, for all the acinous tissue to disappear completely could not be precisely determined, but none was found, by vital staining and careful searching of the whole pancreas, in one rabbit that was kept for 533 days after duct ligation, although considerable islet tissue, along with columns of undifferentiated cells, was present in the mass of fat which occupied the position of the old pancreas (Fig. 4). It may be added that this animal showed no glycosuria. In a rabbit kept for twenty-one months after ligation of the duct (by

have become established at a distance of about 2 centimetres from the stump of the old duct. Through the new duct coloured fluid could be injected from the pancreatic end into the bowel.

employing a combination of vital staining with janus green and vascular injection with carmine gelatine) the islets were found to have the characteristic arrangement of blood vessels, viz., a direct arterial supply and a rich capillary net. These painstaking investigations show that in the cases of the rabbit and guinea-pig it takes at least several months after duct ligation before all traces of acinous tissue disappear. How long it would take in the dog is as yet undetermined.[1] The demonstration of regenerated acini, the cells of which contain zymogen granules, is highly significant.

II. Pancreatic Grafts.—It is the custom, in making pancreatic grafts, to use the vertical or caudal portion (uncinate process), and to preserve the blood-vessels which run to it in the mesentery by which it is attached to the duodenum. When the graft is transplanted into the abdominal wall it is an easy matter, at intervals, to excise small portions for histological examination. When this operation is done in the dog, the most constant change seems to be marked growth of interlobular connective tissue (sclerosis), with varying degrees of degeneration of the acinar and islet cells, that of the former predominating.

Pratt (1912), however, found in six months after transplantation of a graft of the splenic end of the pancreas into the spleen, the rest of the gland being removed, that there still remained unmistakable acini, but no islets. This unusual finding has also been reported by Milne and Peters (1912) in various animals after isolation of the splenic portion. These authors based the identification, as acini, of the collections of cells which still remained after several months, upon the presence of connections, either with ducts or with obviously atrophic acini. Of those reporting the persistence of both acini and islets in the grafts may be mentioned Kyrle (1908) and Tiberti (cf. Allen), who examined transplants into the spleen or liver. Laguesse (" Le Pancréas ") describes changes affecting both acinar and islet cells in a graft transplanted three months previously into the abdominal wall. Dewitt (1906) tied off portions of the pancreas in cats and, in the animals which survived for some time, found that, although much fibrous tissue appeared, islets could always be recognised amongst it, although the acini underwent extensive degeneration. MacCallum (1909) isolated, by ligation, one third of the pancreas in the dog, and when after seven months this part had atrophied, he excised the remaining portion of the gland. Twenty days later the atrophic remnant was removed and found to be composed of cells resembling those of the islets.

[1] In the dog, the extensive development of fibrous tissue obscures the changes in the glandular elements, and it ultimately causes islets, as well as acini, to disappear, probably by interfering with their blood supply (cf. F. M. Allen).

In the foregoing investigations, with the exception of those of Laguesse and Gontier de la Roche, the identification of the islets was based mainly on the absence from the cells of zymogen granules, and not on the presence of the α and β granules, which are now considered to characterise them (p. 4). As has been pointed out by R. R. Bensley, the identification of islet cells was made dependent not on positive, but on negative characteristics; not on what granules they possessed, but on what they did not possess. By such criteria any cells not exhibiting zymogen granules, except those that were obviously duct cells, were considered as islet cells.

Taking advantage of the newer cytological methods, Kirkbride (1912) ligated the distal end of the pancreas in the guinea-pig, and in fifteen months found a much distended duct surrounded by remnants of pancreatic tissue. By Lane's methods of staining, applied to sections of this tissue, she could identify the cells as islet cells, no acinar cells being visible. But even this refinement of technique does not make the results of the duct ligation and the graft experiments entirely satisfactory, incomplete obliteration of the ducts, or their re-growth, orsclerosis, being still possible causes for confusion. There is, nevertheless, almost complete unanimity of opinion that the islets are present, even in a much atrophied tied-off piece of pancreas, at least in the rabbit and guinea-pig, but it is not so certain that some acini may not also be present, for, as Bensley has pointed out, failure to identify these in sections in one plane only is inadequate evidence that they are likewise absent from the entire residue of gland. To show actually whether any acinous tissue is left, it is necessary, according to this authority, to use one of the methods of vital staining (p. 8), and to search the whole gland residue systematically.

III. Pancreatic Remnants.—The problem of the specific anti-diabetic function of the islets has been attacked by another method, introduced by Homans (1913) and F. M. Allen (1913), the principle of which is to remove a sufficient portion of the pancreas to cause the milder symptoms of diabetes, and then to examine the changes in the remaining portion. Under these conditions the diabetic symptoms gradually become more and more severe, or they may be absent at first but develop later, this being favoured by adding carbohydrates to the food. When the

diabetic symptoms were very mild, Homans found that the granules of the β-cells (stained by Bensley's method [1]) became reduced in number. Later (1914), he demonstrated that when the diabetes was severe, decided degeneration of these cells was present, as evidenced by loss of granules, deficiency of protoplasm, known as hydropic degeneration of the β-cells, and decided nuclear changes. On the other hand, the acinar cells were perfectly normal.

Homans also found, after injecting secretin, during a period of nine hours, into a dog (as a result of which 210 c.c. of pancreatic juice was collected) that the zymogen granules had practically disappeared from the acinar cells (leaving the fuchsinophile mitochondrial filaments), but that the islets were unchanged and more sharply marked off than usual from the acini. There was no indication of transition of acinous to islet tissue. After nearly complete removal of the pancreas he describes shrinkage of the acinous cells in the remnant (subcutaneous graft) which remained after several weeks. There was no evidence of conversion of acinous to islet cells, but rather "a suggestion of a relapsing of islet into duct tissue." This close association of the islets to the ducts is emphasised. At the same time, the β-cells of Bensley and Lane were found to tend to lose their granules, and so to appear like duct cells.

Homans inclined to the view that these changes in the islet cells are the result of exhaustion, due to the overstrain put upon them by removal of the greater portion of the pancreas. It would appear that there must be a critical level below which the islet tissue cannot become reduced if it is to survive the strain of providing sufficient internal secretion to prevent the development of diabetes. When the pancreas remnant, whether grafted or left *in situ*, is of such a size that the strain on the islet cells is not very great, as indicated by mild diabetes, degeneration of some island cells may be compensated for by proliferation of new cells from the ducts. On the other hand, when the remnant left after partial pancreatectomy is a small one, and especially when it is composed of the uncinate end of the pancreas in which, as we have seen, the islets are scarce, the constant strain is so great that any new cells that may form soon suffer the same fate as the degenerating older ones.

F. M. Allen (1913), in the same year as Homans, described

[1] The particular technique employed was anilin-acid-fuchsin-methyl-green after osmic-chrome-acetic fixation. The author states that this is particularly satisfactory when the dog pancreas is used.

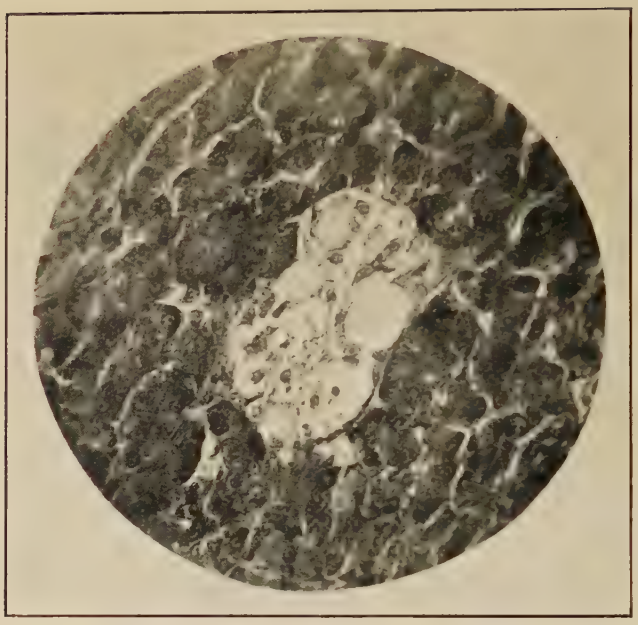

FIG. 5.—Hydropic degeneration of the cells of an islet in a portion of pancreas left *in situ* for fourteen days after removal of the remainder of the gland. (D. J Bowie.)

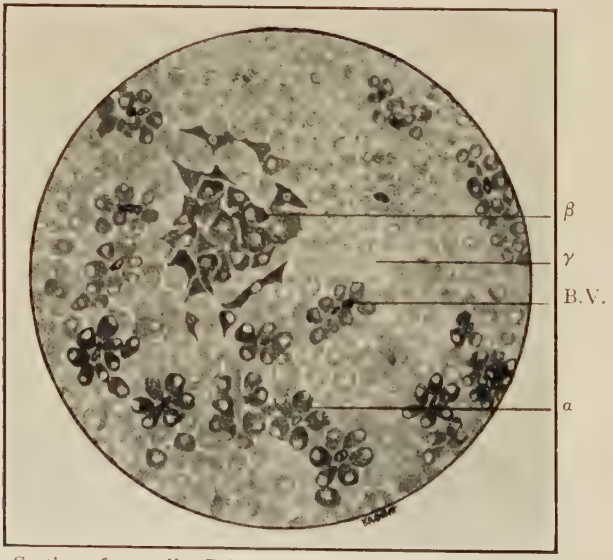

FIG. 8.—Section of a small "Principal Islet" of *Neomænis griseus*, stained by ethyl violet and Biebrich scarlet, × 360. (D. J. Bowie.)

very much the same changes in pancreatic remnants as did this author. By the removal of about nine-tenths of the pancreas in dogs, the remaining portion being left near the duct, he was able to produce a form of experimental diabetes which is characterised by the absence, or the relative mildness, of diabetic symptoms immediately after the operation, followed later, however, by their development in full intensity. By microscopic examination of the remaining fragment (usually stained by eosin and methylene blue), Allen found in very early stages (showing mild diabetic symptoms) no visible changes in the islets, indicating that the diabetes was in one sense functional. At later stages the islet cells were found to be reduced in numbers, many of those remaining being frequently swollen, deficient in cytoplasm, and with degenerating (generally pyknotic) or naked nuclei. In cases of long standing, positively recognisable islets had disappeared. In all cases, on the other hand, the acini remained normal. Allen (1922) has since extended these observations, and has clearly demonstrated, by a series of excellent photomicrographs, that it is the β-cells of the islets, and not the α-cells, which become involved. In Fig. 5 is shown a microphotograph of a very small remnant of pancreas left for fourteen days after removal of the gland itself. Marked hydropic degeneration of the insular cells is clearly present (D. J. Bowie).

W. B. Martin, by the use of special stains (neutral ethyl violet azo-fuchsin, or neutral ethyl violet-orange-G), describes four stages as follows: (1) The β-cells appear swollen and more sharply defined, with a thinning out of the granules; (2) vacuolation of the β-cells, without nuclear changes; (3) shrinkage of the nucleus and breakdown of the cell body; (4) disappearance of the β-cells.

It must be pointed out, in connection with the work of Homans and Allen, that the hypothesis, that overstrain of the remaining islet cells is responsible for the changes which occur in them, is not supported by any direct evidence. There is no other animal tissue, so far as we are aware, in which structural changes, similar to those observed in the islets, occur as the result of overstrain.

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CHAPTER III.

THE ISLETS IN FISHES AND THEIR YIELD OF INSULIN.

Comparative Anatomy.—It would be impossible here to give a full account of the numerous investigations that have been made concerning the comparative anatomy of the islet tissue, but it is important to pay some attention to those that deal with its distribution in fishes.

In 1846 Stannius described, as blood glands, structures in the abdominal cavity of certain of the Teleostei, and in various other lower vertebrates. These were afterwards shown by Diamare (1899) to be homologous with the islets of Langerhans of the higher vertebrates. He pointed out that one can frequently find two or three of them in the neighbourhood of the liver and spleen, as well as several smaller ones just visible to the naked eye implanted in thin and poorly developed pancreatic tissue. In certain fishes, such as *Lophius piscatorius* and *Scorpæna scropha*, these cell groups have a definite capsule which apparently separates them from the pancreatic tissue, and they may be of such dimensions that they can easily be removed.

Diamare (1904) clearly recognised that the cells composing these structures are homologous with those of the islets of Langerhans of the pancreas of higher vertebrates, and he attempted to show that they must produce an internal secretion capable of influencing the metabolism of carbohydrate substances. For this purpose, the large islets of Lophius were ground up with glass powder and extracted with distilled water. The extract was not found to influence the rate of hydrolysis of a boiled starch solution, whereas, in control experiments in which the extract was prepared from pancreatic tissue, diastase could easily be demonstrated by the same methods. In another series of experiments, in which extracts of the islets from Scorpæna were used, some indications of slight glycolysis in a solution of dextrose were obtained. In a later communication (1905), Diamare states definitely that the islets of Langerhans have an endocrine function in connection with the metabolism of glucose

in the body, and that hyperglycæmia and diabetes are associated with their inadequate functioning. Previous to Diamare's work, reference to possible islet tissue in the eel, *Anguilla vulgaris*, was made by Massari (1898), who also described two kinds of cells, chromatophile and achromatophile. Further work on lower vertebrates will be found recorded in the monograph by Laguesse (1906).

Notwithstanding the extensive literature that had accumulated, there was, in 1904, considerable disagreement as to the real nature of the structures referred to, but in this year John Rennie published an important paper, entitled "The Epithelial Islets of the Pancreas in Teleostei," which strongly supported Diamare's views. Rennie examined the islets in twenty-five species of Teleostei, and usually found at least one encapsulated islet, which he called the "Principal Islet," of relatively large size and of constant anatomical position in each species of fish.[1] In some species only one such body was found, but in others there were several, of which one was always larger than the others, which were not constant in number. By comparison of different fishes, Rennie was able to distinguish various degrees of intimacy between the insular and the zymogenous cells, and he concluded that the principal and other separate islets [2] are directly related to the pancreatic islets of the mammalian pancreas. He pointed out also that sometimes pancreatic elements (zymogenous tissue) penetrate the islets, the two kinds of cells being separated by connective tissue. It is interesting that in certain fishes, such as *Pholis gunnellus* and *Zoarces viviparus*, pigment which is contained in the peritoneum and along the mesenteric blood-vessels is also present in the capsules of the islets. The unusually rich blood supply of the separate islets is alluded to, but there is no detailed description of the cytological characteristics of the cells. Rennie was thoroughly convinced that the islets have an internal secretory function, and in a later paper, along with Fraser (1907), an account is given of attempts to demonstrate this by seeing whether extracts of the islets could affect the rate of glycolysis of sugar solutions, or could remove the symptoms of

[1] He had previously reported on this work in 1903, after examining six types of fishes.

[2] For brevity we will designate as principal islets both the principal and the other separate islets of Rennie.

diabetes in man. This latter work has been referred to elsewhere (p. 56).

It can be seen from the preceding brief review that, as a result mainly of anatomical investigation, there is much evidence that the principal islets of the Teleostei are homologous with structures which exist in the mammalian pancreas as the islets of Langerhans. Before this identity could be established, however, it was necessary to determine, first, whether the cells of both these structures were possessed of the same cytological characteristics ; and, secondly, whether they bore towards carbohydrate metabolism the same functional relationship. With regard to the cytological characteristics of the cells, it may be said that at this time, 1904, histological methods had not been evolved in sufficient detail to demonstrate clearly a fact now well known, namely, that there are in the islets of Langerhans at least two types of cells, neither of which appears to be related to the zymogenous cells. It is true, as we have seen elsewhere, that various observers, including Laguesse and Diamare, had recognised that two types of cells were present, but without giving clear directions by which they could be differentiated with certainty, and it was only after the work of Lane (1907) that this differentiation came to be definitely established, the two kinds of cells being designated α and β respectively.

Taking advantage of these methods, the histological investigation of the islets in fishes was again taken up in 1922, by Slater Jackson, who was able to differentiate α- and β-cells in the Elasmobranch pancreas (in Raja) (p. 13), and then to show that these two kinds of cells also exist in the principal islets of Teleostei (*Pholis gunnellus*). Some ninety species of fish were subsequently examined by N. A. McCormick (1924), principal islets being found in the vast majority of instances. Considerable variation was shown to exist among different species, with regard to the position, size, and number of the islets, and interesting facts were brought to light regarding the relationships of the zymogenous to the islet tissue. Various types of distribution were observed among fishes of different orders, and while no general rules could be laid down, a certain degree of association between the form and location of the islets and the accepted classification of fishes was evident.

In making these comparisons, it is advantageous to recall

that the islet tissue present in the pancreas of Elasmobranchii is in the form of fairly large irregular masses (see Fig. 2), imbedded throughout a compact, definitely outlined pancreas, as in mammals, and plainly in epithelial continuity with the duct system of the gland. In the ganoid fishes, whose divergence from the Elasmobranch type is not very great, the pancreas is studded with islets and, although fairly compact, it tends to spread out and surround the portal vessels, without following them into the liver. In the lower orders of Teleostei, the islets are imbedded in masses of zymogenous tissue, which seems to become more diffuse as the scale is ascended and tends to invade other tissue, particularly that of the liver. In the Lake Catfish (*Ameiurus lacustris*), McCormick and Bowie observed what appears to be one of the steps in the process of isolation of the islet tissue from that of the pancreas. Numerous small (1 mm. in diameter) discrete, greyish-white, oval bodies are present in the mesentery, and each was seen on section to be made up of a core of islet tissue, without any fibrous capsule, but intimately surrounded by a thick layer of zymogenous tissue. A single, large islet extensively invaded by bands of the zymogenous tissue is also present. In the Pollock (*Pollachius virens*), which belongs to a higher order, further isolation of the islet tissue becomes evident. Gross examination revealed this tissue in one large, somewhat flattened body in the mesentery, attached to the side of the gall bladder. Occasionally it was seen to be divided into two parts. While the mass is composed mainly of islet cells, it is wholly surrounded by zymogenous tissue, which penetrates it everywhere. The impression which a section gives in this case is of islet tissue invaded by branching rows of zymogenous cells springing from a thick capsule composed of the same tissue. Similar relationships of islet to zymogenous tissue were revealed in a large number of common fish, such as the Hake (*Merluccius bilinearis*), Cod (*Gadus callarias*), and the Haddock (*Melanogrammus aeglefinus*). In many other fish, such as *Lophius piscatorius*, *Myoxocephalus*, *Pholis gunnellus*, and *Pseudopleuronectes americanus*, similarly encapsulated islets exist, apparently uninvaded by zymogenous tissue, the most complete isolation of zymogenous tissue being perhaps that found present in *Zoarces* and *Myoxocephalus*. A full account of the relationship of islet to zymogenous tissue will be found in the paper above referred to by McCormick.

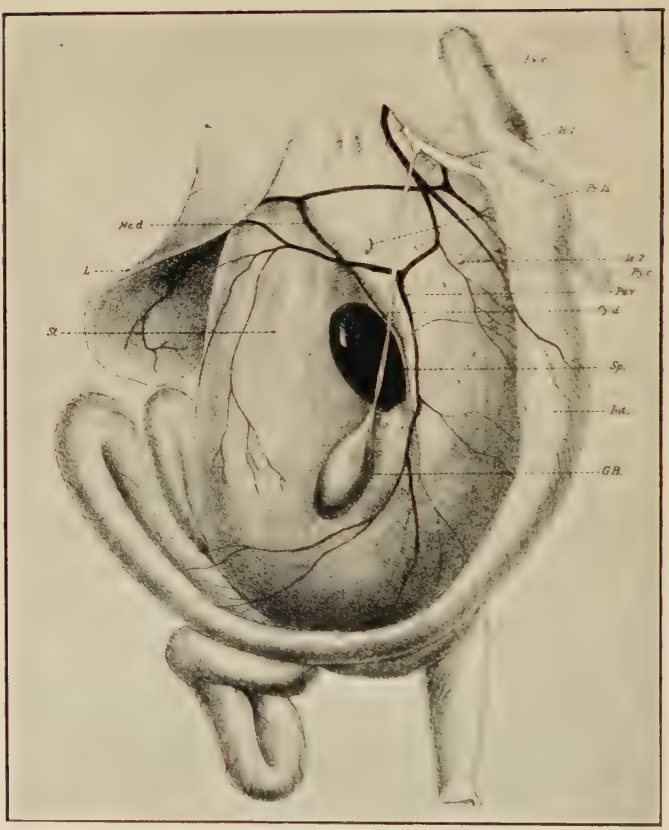

FIG. 6.—The position of the "Principal Islets" in *Lophius piscatorius*. The letters indicate the various anatomical structures. (From J. Rennie.)

The Yield of Insulin from Fish Islets.—It was shown by Macleod (1922) that it was possible, by alcoholic extraction, to obtain large yields of insulin from the principal islets of *Lophius piscatorius*, of *Myoxocephalus*, and of other Teleostei, whereas very little, if any, could be extracted from the zymogenous tissue. In *Lophius* (see Fig. 6) two large islets exist, one of them being constantly present, as described by Rennie, in the mesentery in the neighbourhood of the spleen, where it lies near the conspicuously long bile duct. This, the largest islet, may weigh about half a gram. Another slightly smaller one lies nearer the pylorus, accompanied usually by several minute ones, scattered in the upper mesentery. Pancreatic tissue exists, as bands of zymogenous tissue, surrounding tributaries of the mesenteric and portal veins, and in this no islets can be distinguished by the naked eye, at least in that part present in the lower mesentery, although on microscopic examination Slater Jackson has detected occasional small collections of islet cells.

To illustrate the remarkable effects of an extract from the principal islets, the following results are given:—

From a total of 1·15 gm. moist weight of islets 12 c.c. of extract was obtained after removal of the alcohol, and 2 c.c. of this, injected into a rabbit of average weight, caused the blood sugar to fall from 0·108 per cent. to 0·042 per cent. in an hour and to 0·024 per cent. in two hours. The rabbit, meanwhile, developed typical convulsions which, although temporarily relieved by the injection of glucose, reappeared two hours later when the blood sugar was found to have fallen to 0·025 per cent. A second injection of glucose restored the animal to normal.

In subsequent experiments, in which large quantities of islets obtained from *Lophius* were extracted, much larger yields of insulin were obtained, and after Berkefelding this was used by Dr. W. R. Campbell in the treatment of diabetes in man with entire success.

In the Sculpin (*Myoxocephalus*) there are usually two principal islets, the largest and most constant of which is situated in the mesentery immediately anterior to the spleen and close by the portal vein, the smaller one being near the pylorus, where there are also, usually, several just visible islets scattered here and there in its neighbourhood.

An extract of these islets (weighing *in toto* 0·58 gms.), measured 12 c.c. after removal of the alcohol, and 1 c.c. of it injected into a rabbit

weighing 2·25 kg. lowered the blood sugar in about an hour from 0·092 per cent. to 0·042 per cent. Convulsions and profound coma then developed and the blood sugar fell almost to vanishing point, 0·010 per cent. Injection of glucose restored the animal temporarily, but had to be repeated to bring it back to a normal condition.

In both *Lophius* and *Myoxocephalus*, extracts prepared in exactly the same manner from large quantities of pancreatic tissue which had been dissected from the lower mesentery of several fish had very little, if any, effect on blood sugar, even when the whole extract was injected.

Thus, all the pancreatic tissue from two specimens of *Lophius* gave an extract which was finally evaporated to a volume of 5 c.c., all of which was injected into a rabbit weighing 2·4 kg. One hour after injection the blood sugar stood at exactly the original level (0·124 per cent.), although in an hour later (that is, two hours after injection) it had fallen to 0·110 per cent. In another case in which the pancreatic tissue of *Myoxocephalus* was used, the extract produced a slight increase in blood sugar, namely, from the normal of 0·110 per cent. to 0·118 per cent. in about an hour after injection, rising later to 0·124 per cent. in about three hours, and 0·156 per cent. in about four hours.

Strong evidence was furnished from these results in favour of the hypothesis that insulin, as its name implies, is derived from the insular and not from the zymogenous tissue of the pancreas. Further investigations have shown that from the islets in the cod, hake, and haddock, notwithstanding the associated zymogenous tissue, very large yields of insulin can readily be prepared, both by the alcohol and the picric acid methods. Indeed, the yields were so large that a thorough investigation was undertaken by McCormick and Noble (1924) of the possibility that insulin might be extracted for commercial purposes from this material. Insulin of high purity was prepared on a large scale with comparatively little expense from the islets, particularly of the cod. Thus in one observation, from a total weight of 22,000 lb. of fish, 2400 clinical units of insulin were obtained—that is, 100 units per 1000 lb. of fish—at a cost of less than 0·4 cent per unit. McCormick and Noble, as the result of a thorough investigation of the commercial possibilities, and taking into consideration all the difficulties of collection of material by unskilled labour, came to the conclusion that fish should prove a profitable source of manufacture in countries where the fishing industry is large and is continuous throughout

the year. The general relationship of the islets to the gall bladder in the commoner fishes is shown in Fig. 7.

Cytological Characteristics of Fish Islets.—The most complete account of the cytological characteristics of the cells composing the principal islets of Teleostei is that of D. J. Bowie (1924). Diamare, Laguesse, and Rennie had already described these characteristics, as far as they could be studied by use of the methods of staining available when their work was done, and they, as well as Slater Jackson, who used more modern methods, obtained evidence that the cells are homologous with those of the islets of Langerhans of the mammalian pancreas. Bowie studied the principal islets found in the grey snapper (*Neomænis griseus*), fixing them in a modification of Zenker's fluid (with 2 per cent. acetic acid), and staining mainly by a neutral stain

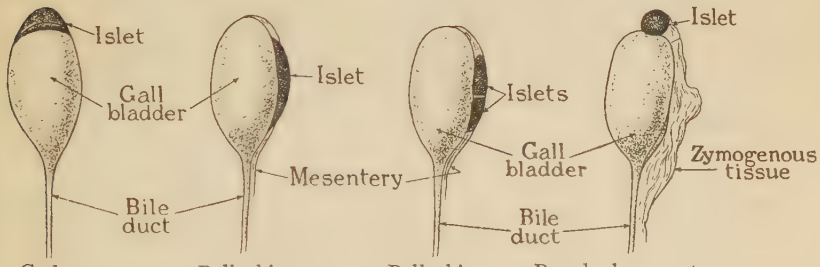

FIG. 7.—The position of the " Principal Islets " in relationship to the gall bladder in some of the common fishes. (McCormick.)

which he compounded from ethyl violet and Biebrich scarlet (disulphonate of Sudan iii.). Medium-sized islets were found to be the most useful for study. By low-power examination a capsule is readily visible, and lying outside it appear scattered, narrow strands of zymogenous cells, the granules in which stain very darkly and are large. From the capsule of the larger islets, trabeculæ may proceed inwards among the islet cells and somewhat confuse the picture, but in the smaller ones, where there are no trabeculæ, a more or less irregular central zone composed of densely granular cells is seen to be surrounded by a peripheral zone of less granular ones.

As a result of closer examination under the high power, in sections stained preferably by the Biebrich scarlet stain, but also in those stained with eosin methylene blue, or with neutral gentian violet following alcohol-chrome-sublimate fixation,

Bowie concluded that the cells of the central zone are homologous with the alpha cells of the islets of the mammalian pancreas, whereas those of the peripheral zone consist of two varieties, one being homologous with the beta cells, and the other representing a variety of cells (gamma), which are either not demonstrable or are scanty in mammalian pancreas (Fig. 8).

The *alpha* cells are tightly packed with granules which stain blue with Bowie's stain, or with eosin methylene blue or neutral gentian violet. They vary considerably in numbers in different islets, being sometimes absent in very small ones. When stained with neutral gentian, after fixation in the alpha cell fixative of Lane (p. 5), these cells alone are tinted blue. The alpha cells are often arranged in twisted columns, each cell being more or less pear shaped, with the narrower end abutting on a blood-vessel. When one of these columns is seen in transverse section, a rosette arrangement is evident, the small blood-vessel occupying the centre of the rosette. The nuclei are in the wider end of the cells, away from the capillary, being often oval in outline, with the long axis at right angles to that of the cell. Sometimes all the cells surrounding a blood-vessel are not of the alpha variety, being intermixed with other types, and especially in larger islets the relationship of the alpha cells to the blood-vessels is not so evident.

The *beta* cells, which in the mammalian pancreas are believed to be the source of insulin (p. 24), stain, in the principal islets, purple with Bowie's stain and brick-red with eosin methylene blue after the modified Zenker fixation. They are not so densely granular as the alpha cells and they occupy either a peripheral position, or one next the trabeculæ, when these are present. They are more irregular in shape than the alpha cells, and long processes may extend from them between the other (gamma) cells. Although not, as a rule, so characteristically arranged as the alpha cells, grouping may occur in certain islets to form compact areas of beta cells. Curiously enough, the beta cells are less numerous than alpha and gamma cells, and they are not usually clearly related to blood-vessels. The granules are somewhat larger than those of the other cells of the islet and some may, indeed, reach almost to the size of small zymogen granules which might induce one to consider them a transitional form of acinar cells. The nucleus is uniformly round, with an oxyphilic nucleolus in the centre.

The *gamma* cells, which may be the same as those described by Bensley in the mammalian pancreas (guinea-pig), stain a bright red colour with Bowie's stain and with eosin methylene blue. They are largely responsible for the lighter appearance of the peripheral zone of the islets. The outlines of the cells are less well defined than those of the other varieties to which also the cells may bear various relationships, being either evenly scattered among them or collected into loosely packed masses in which blood-vessels are not conspicuous. The nucleus occupies a central position in the cell, is spherical or elliptical in outline, and has one or more small nucleoli.

Apart from the encapsulated islets, such as those which have just been described, very small non-encapsulated collections of islet cells may also be seen scattered among the zymogenous cells. As a result of the careful examination of a large number of preparations, Bowie was never able to obtain any evidence of a transition between islet and acinar cells such as has been described by Laguesse. He believes that the islet cells are derived from the epithelium of the smallest ductules.

In order of their appearance, Bowie states that in the smallest islets gamma cells are mostly present, with perhaps a few beta cells. As the islets increase in size the beta cells become more numerous, and then the alpha cells appear towards the centre. Contact between alpha and beta cells is rare, and it is possible that the gamma cells, which intervene between them, may be the source of the alpha, if not also of the beta cells. Bensley has also suggested that the gamma cells described by him in the pancreas of the guinea-pig may be the source of the alpha cells.

The Effects following Removal of the Islets (Isletectomy).— In the Sculpin (*Myoxocephalus*) it is an easy matter to excise the two largest principal islets without touching the pancreatic tissue.

For this purpose, fish of an average weight of one kilo are chosen, and after wrapping them in wet towels, the abdomen is opened, the two principal islets exposed by retraction, the tissues in which each lies mass ligated, and the islets excised. The whole operation, including stitching of the abdominal wound, occupies about fifteen minutes, and the fish immediately swim about in normal fashion when replaced in sea water.

By determination of the sugar in blood, aspirated directly from the heart, of fish removed at varying daily intervals, McCormick and I (1924) found hyperglycæmia of high degree to exist for as long as eleven days following the operation. This does not, however, justify the conclusion that the isletectomy is the only cause of the hyperglycæmia, since we have found, in other fish, that such exposure to air as is entailed in the operation, will in itself cause marked hyperglycæmia, no doubt because of asphyxia. But this asphyxial hyperglycæmia has seldom been found to last for more than five days at the most (see p. 234), and, even during this period, the blood sugar is on an average not nearly so high as in the isletectomised fish. The results of

these observations are shown in the chart of Fig. 9, in which the white vertical columns give the percentages of sugar (indicated on the ordinates) found in the operated fish, and the black ones, those in controls on the days following the operation (indicated on the abscissa). It may be mentioned that in other studies of the duration of the effects of asphyxia in *Myoxocephalus*, the blood sugar was always found to have returned to the normal (between 0·010 and 0·035) within four days at least.

Simpson has more recently (1925) proved beyond all doubt that the hyperglycæmia is dependent on the isletectomy, and further evidence that this symptom in fish, as in mammals, is

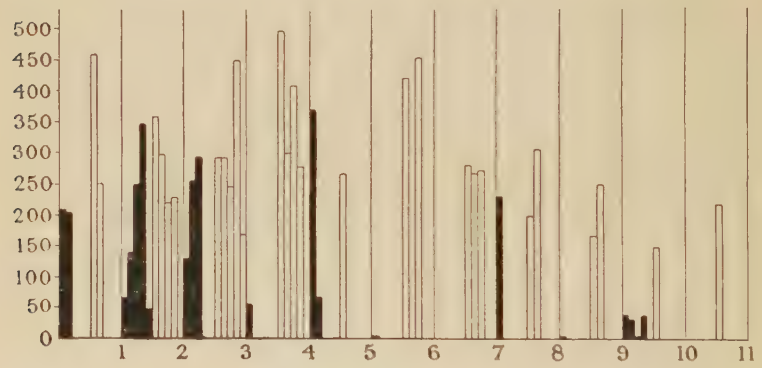

FIG. 9.—White columns represent milligrams per cent. of blood sugar in specimens of *Myoxocephalus* removed on various days following isletectomy. Black columns represent percentage of blood sugar in control fish. Percentage of blood sugar is given vertically. Days after isletectomy are indicated on base-line.

accompanied by the others that characterise diabetes, is furnished by the fact that the livers of the isletectomised fish were found to contain decidedly less glycogen and more fat than those of the controls. This is shown in the table on page 37.

Taken in conjunction with the fact that much larger yields of insulin can be readily prepared from the principal islets than from the pancreas itself in *Myoxocephalus*, as well as in *Lophius* (p. 31), there can be little doubt that the islets are its source. The smaller quantities said to occur in other tissues are probably carried to them by the blood and stored up there. In any case, it has never been shown that excision of any other tissue than

Isletectomy.			Controls.		
No.	Fat Per Cent.	Glycogen Per Cent.	No.	Fat Per Cent.	Glycogen Per Cent.
259	19·4	0·54	166	5·7	3·30
198	21·8	0·30	168	12·8	0·10
165	29·4	0·14	287	14·3	trace
146	35·3	0·30	130	15·2	0·19
157	37·4	1·34	136	26·8	6·36
117	42·0	trace	142	27·6	0·05
151	43·0	0·20	276	32·7	0·24
140	54·0	0·56			
Av.	35·3	0·42		18·6	1·46

the pancreas of mammals, or the principal islets of fishes, can cause persistent hyperglycæmia. Of course, to complete the chain of evidence supporting the insular hypothesis, it would be desirable to ascertain whether injection of insulin (derived from the principal islets) into isletectomised fish would reduce the hyperglycæmia, and experiments in this direction are under way.

Review of Preceding Evidence that Insulin is Derived from Islets.—It has been considered by various investigators that the islets may be derived directly from the acini, and indeed it was at one time suggested that the former are nothing more or less than groups of exhausted zymogenous cells. In support of this view, some have thought that an increase in the relative numbers of islets could be demonstrated when the external secretory function of the gland was persistently stimulated by secretin (H. H. Dale), or even in the opposite state, when this function was suppressed, as after prolonged starvation (Swale Vincent and Thompson). These researches were undertaken prior to the work of Lane and R. R. Bensley, in which it has been shown that there is no basis for such a view, when identification of the islet cells is made dependent, not merely on the absence of zymogenous granules, but rather on the presence in them of specific granules, and on other cytological characteristics. As has been pointed out by Bensley, the importance of a final settlement of this question is much greater than if it referred solely to the function of the pancreas in its relationship to the metabolism of the carbohydrates, for if the two types of cell represented nothing more than the same anatomical structure in different stages of activity, it would be a challenge to the

doctrine that differences in the structure of cells is evidence of essential differences in function. It is true that a third possibility exists, namely, that the same cells might functionate as the source for external secretion at one stage, and for internal secretion at another. Such a view has been defended by Laguesse. He supposes that there is a vicarious relationship between the acini and islets, and, as evidence, he describes types of cell which are transitional between those of the two structures. Presumably, when the demands for insulin are increased, insular cells become developed at the expense of the acinar, and *vice versa*. The acinar cell, according to this view, does not secrete both insulin and pancreatic juice at the same time, but it may give up the one function, become changed in its cytological structure, and thereby assume the other function. Three entirely different views, therefore, are, or have been, expressed with regard to the physiological anatomical relationships of the pancreatic cells: (1) That all are concerned in the same functions; (2) that there are two groups that are entirely different both in structure and function; (3) that one kind may change into the other with regard to both structure and function.

The studies in the comparative anatomy of the islets, the yield of insulin obtainable from them, and the effect of excision of the islets reviewed in this chapter, afford very strong evidence that acini and islets are as distinct and separate from each other, both anatomically and physiologically, as are the anterior and posterior lobes of the pituitary gland, or the cortex and medulla of the adrenal, or the parathyroid and thyroid glands. This evidence may be summed up as follows:—

1. The insular tissue in certain of the Teleostei, *Myoxocephalus* or *Lophius* for example, is entirely surrounded by a definite capsule, and if any zymogenous cells happen to be located near this principal islet, they are manifestly quite separate and apart from the cells which compose it.

2. In other fishes (*Pollachius*, *Gadus*, etc.) a considerable amount of zymogenous tissue may be seen in juxtaposition with the principal islet, but it is either outside its capsule or separated from the islet cells by the fibrous tissue which composes the trabeculæ, along which the bands of zymogenous cells extend into the islets.

3. By very careful searching of sections, fixed and stained by various methods developed to differentiate islet cells, of the pancreas of animals in which there is apparently a close relationship of islets and acini, Bowie and others have been unable to confirm the statement of Laguesse, that forms of cell transitional between those of islets and acini can be seen.

4. When simple, acid alcoholic extracts are made of the islets of *Myoxocephalus*, or *Lophius* or *Pseudopleuronectes*, in which there are only traces of zymogenous cells, the yield of insulin is very large indeed (p. 31), whereas in similar extracts of the practically islet-free zymogenous tissue of these same fishes no insulin, or only comparatively small yields, are obtained. This does not in itself, however, prove that no insulin is present in the zymogenous tissue, for it may either have become destroyed by the digestive enzymes, or the methods of extraction, though satisfactory for the islets, may be unsuitable for the zymogenous tissue. As a matter of fact, Swale Vincent, Dodds, and Dickens have obtained, by use of the picric acid method (p. 71), from the zymogenous tissue of *Lophius*, yields of insulin which are one-fifth to one-seventh (weight for weight of tissue) of that from the islets, and large amounts have also been prepared by them, and by others (see p. 73), from such glands as the parotid, the testes, etc., of mammals. But this does not materially weaken the evidence, for it is admitted by these workers that by far the largest yields of insulin are derivable from those principal islets to which are attached only small groups of zymogenous cells. Insulin, possibly in a physiologically inert form, may be present in most of the active cells of the body (see p. 89), but it is only stored there temporarily; it is manufactured, so to say, in the islet cells, but traces may be found in other tissues to which it has been carried by the blood.

5. When the two principal islets are removed in *Myoxocephalus* the blood sugar rises to more than ten times the normal value, and remains at this high level for as long as the fish can be kept alive (up to twenfy-one days). This greatly exceeds the period during which asphyxia could be held accountable for the disturbance in carbohydrate metabolism (p. 234).

It is true that, embryologically, the acinar and the insular cells are derived from the same "anlage," but it has proved to

be very difficult to trace the development throughout all its stages. R. M. Pearce, and more recently W. C. M. Scott, have contributed much precise knowledge in this field. The latter has shown in *Myoxocephalus* and *Pseudopleuronectes* that the embryological outgrowth, which ultimately forms the principal islets, appears as a definite structure at a very early stage, and remains apart from those cells which afterwards become zymogenous. Both kinds of cells have apparently the same embryological source, but once differentiated, each develops in its own way, and cannot thereafter become reconstructed into the other type. It is possible that vestigial remnants of the original mother cells may remain throughout life, perhaps in relationship with the ducts or ductules, and that new insular or acinar cells may develop from them; and it is interesting to consider the possibility that such development may be stimulated when an urgent call arises within the body for more of the particular secretion which the cell produces. Thus, when the internal secretory function of the pancreas is greatly crippled by morbid destruction of the islet cells, it is possible that new ones may become differentiated from the duct epithelium, and thus restore, in part at least, the failing function.

Comparison of the distribution of islet tissue in the pancreas of the Elasmobranchi with that in the pancreatic residue remaining in mammals several months after ligation of the ducts (p. 21), affords some support for the possibility of regeneration of the islets. It is unfortunate, however, that these investigations can only be made satisfactorily on rabbits and guinea-pigs. In these animals it is difficult to conduct metabolic studies, and, in the case of the rabbit, it is not possible to remove a sufficient portion of the gland so that diabetes occurs.

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CHAPTER IV.

PANCREATECTOMY.

Although various of the earlier investigators, prompted by the repeated clinical reports that a pathological condition of the pancreas is common in severe diabetes,[1] had tried to produce this condition in animals by extirpation of the pancreas, no successful results were published until 22nd May, 1889. On this date von Mering and Minkowski announced their well-known discovery, and in 1893 Minkowski described in detail the technique of the operation of removal of the pancreas, along with a complete account of the diabetic condition which supervenes. Between the preliminary announcement (in 1889) and the final paper (in 1893), there appeared two others to which brief reference will be made, since they indicate the dates of several important discoveries. They appeared as lectures in 1890 and 1892, and in them Minkowski pointed out that, since partial extirpation of the pancreas causes no diabetes provided at least one-tenth of the gland is left in the body, neither injury to neighbouring nerve structures, nor removal of the intestinal secretion of the gland can be responsible for its occurrence. In one dog from which nine-tenths of the gland had been removed, a mild form of diabetes resulted, the animal excreting sugar when fed on carbohydrates, but not so when fed on meat and milk. It is to the investigation of the experimental diabetes of this type that Sandmeyer (1895) and, later, F. M. Allen (1913), have paid attention. Minkowski attributed the want of success of certain workers who had attempted to confirm his results, either to incomplete extirpation of the gland, or to failure of the animal to survive the immediate effects of the operation, and

[1] Good historical reviews of the earlier experimental work will be found in the monographs of Lombroso (1910), Lépine (1909), F. M. Allen (1913), Warthin (1922). The pathological and clinical aspects of diabetes are reviewed by Opie (1910), MacCallum (1921), Joslin (1923), Graham (1923), Maclean (1923), and Campbell (1924).

he showed that the reputed production of diabetes, after excision of the salivary glands, was due merely to a transitory post-operative glycosuria.

The significance of a constant D : N ratio of 2·7–2·8 in starved, or meat-fed animals, as indicative of sugar formation from protein, was pointed out, and the suggestion offered that disappearance of sugar from the urine at later stages in depancreatised animals might be due to a decrease in its formation from protein. The possibility that any other organ than the pancreas can develop an antidiabetic function was denied. In comparing the diabetes following pancreatectomy with that due to phlorhizin, the important fact was brought to light that when phlorhizin is given to a depancreatised dog, not only does the total amount of sugar excreted increase, but this increase may be in excess of that of nitrogen, so that the D : N ratio rises above the diabetic level, a result which he attributed to a greater permeability of the kidney towards sugar. In discussing the essential cause for pancreatic diabetes, Minkowski concluded that removal of some special function of the pancreas must be responsible, and in support of this view he referred to the experiments first performed by himself, in which, after transplanting a graft of the pancreas into the abdominal wall, excision of the main gland was not followed by diabetes, which, however, set in immediately the graft was excised. In the light of these results, Minkowski considered it highly improbable that it can be because the pancreas acts locally on the sugar of the blood while this is circulating through it, that its antidiabetic function is due, and he concluded that this gland must secrete something into the blood which influences the utilisation of sugar elsewhere in the body, thus clearly stating the theory of the internal secretory function of the pancreas without, however, endorsing the hypothesis, previously advanced by Lépine, that this internal secretion acts as a glycolytic ferment (p. 194).

In the report of his researches appearing in 1893, which is one of the finest papers ever published in the field of medical science, a full and critical account of his own researches and those of others will be found.

After describing the operative methods for complete and partial pancreatectomy in the dog and the pig, he states that pancreatectomy is impossible in the rabbit, because the pancreas is spread out in the

mesentery. The operation is simple in birds (pigeons and geese), but no glycosuria follows, at least in those of herbivorous habits. In carnivorous birds, on the other hand (hawk), Weintraud is reported as having observed that both hyperglycæmia and glycosuria become established. In frogs doubtful results were obtained.

In reviewing the observations on the D : N ratio, Minkowski points out that deviations from this value are not uncommon, and he gives, as examples, that it only rises gradually to 2·8 after pancreatectomy in dogs previously ill-fed, whereas in those well nourished, it may be above this level for a few days. The body stores of glycogen are the source of this early sugar, and the slight variations in the ratio which occur after these become exhausted are to be accounted for by unequal speeds of excretion of the sugar and nitrogen derived from the protein. This irregularity in the D : N ratio, along with faulty absorption from the intestine, makes it difficult to compute what proportion of sugar fed to the diabetic animal reappears in the urine. In carrying out such an observation, Minkowski recommends that the animal should be kept on a meat diet until the daily D : N ratio is constant before the sugar is given. From the total amount of sugar excreted is then deducted the amount which would have been derived from protein, as calculated from the previous D : N ratio. It is evident that if any protein-sparing action occurs, this calculation will not be accurate, and since the belief that the tissues in diabetes have lost the power to utilise carbohydrate is based in part on the results of Minkowski's experiments, it is important to consider some of his results here.

In the first observation, the D : Urea ratio before giving sugar was 1·7; after the ingestion of 15 gms. glucose, 34·5 gms. sugar and 11·8 gms. urea were excreted; therefore, $34{\cdot}5 - 11{\cdot}8 \times 1{\cdot}7 = 14{\cdot}4$ gms. of the 15 gms. administered reappeared. On the next three days, however, by similar methods of calculation an excess of nearly 6 gms. appeared. In the second observation, the D : N ratio was 2·74. After giving 18 gms. glucose the urine contained (that day) 30·4 sugar and 4·44 gms. nitrogen; therefore, $30{\cdot}4 - 4{\cdot}44 \times 2{\cdot}74 = 18{\cdot}2$ gms. re-appeared. But again on the next day, while on flesh alone, an excess of 4·1 gms. sugar was excreted. The protocolls of the third and fourth experiments are either not sufficient for calculation of the proportion of sugar reappearing or are not dependable because of the development of diarrhœa. In neither of these two experiments was all of the administered sugar recovered in the urine.

Minkowski concludes that no significant amount of glucose can be utilised by the animal at the height of diabetes, and he does not comment on the fact that the amount excreted was *actually greater* than that added to the food, when the urine of the day or two following the administration was included. In discussing the cause for the fall in the D : N ratio which commonly occurs as the animal becomes weak, he gives evidence to show that sometimes glucose given in the food fails to reappear *in toto* in the urine, but he nevertheless inclines to the view that it is to a disturbance in the production of sugar out of protein (gluconeogenesis), rather than destruction of part of that formed, that the falling off of the sugar excretion is due.

The numerous investigations on partial pancreatectomy and on pancreatic grafts, already referred to elsewhere (p. 22), are again given in detail in the 1893 paper, partly in order to show that all grades of the disease, as met with in diabetes mellitus in man, can be duplicated experimentally, and partly to throw some light on the question, whether there is any relationship between the external (digestive) secretion of the pancreas and its internal secretion. He agrees with Thiroloix that there is no connection between the two secretions. A large external secretion from the graft might, for example, dry up after a while without causing any change in the diabetic condition.

Definite development of diabetic symptoms are shown to follow when the branches running from the duodenal blood-vessels to the subcutaneous graft of pancreas are clamped, indicating that the new vascular connections between the graft and the parietal vessels are not adequate to convey sufficient internal secretion into the organism.

An average D : N ratio of 2·8, as obtained in completely depancreatised dogs, falls far short of that which would be obtained were all the carbon of protein converted into sugar. Is it possible, therefore, that some of the sugar formed from protein is utilised in the body? To throw light on this problem, Minkowski [1] gave phlorhizin to three depancreatised dogs. Two of them were already very weak, and they, as well as a third one in better condition, did not survive the administration more than a day or two. During the time the animals lived, however, relatively more sugar was excreted than nitrogen, which would

[1] Minkowski here refers to the experiments in which the reappearance of administered sugar was measured as yielding not entirely conclusive results.

seem to show that it is not all excreted after pancreatectomy. Two further points are noteworthy regarding the action of phlorhizin, namely, that after the first (but not the second) dose the increase in the excretion of sugar preceded that of nitrogen, so that the D : N ratio rose higher than that theoretically possible from protein. Removal of either the salivary glands or the duodenum caused only transitory glycosuria, and when the pancreas was extirpated at a later stage, the diabetes was of the usual type.

In investigating the effect of the addition to the food of various carbohydrates, other than glucose, the most interesting result was that obtained with fructose. When 15 gms. were given, only about 0·4 gm. reappeared in the urine, with a doubtful increase in glucose. With larger amounts of fructose much more of it appeared in the urine along with glucose. When inulin, the polysaccharide of fructose, was given, about one-half of the possible fructose was excreted as glucose. Fructose is therefore partly utilised, partly converted into glucose, and partly excreted unchanged. Sucrose and lactose, similarly administered, caused increased excretion of glucose alone.

In animals killed in from three to twenty-six days following pancreatectomy, traces only of glycogen were found present in the liver, even when the animal had been given bread or glucose (four cases). After the ingestion of fructose, on the other hand, considerable quantities of glycogen were found in the liver in one case, and a moderate amount in another. Hédon (1891) also failed to find glycogen in the liver in five dogs following complete pancreatectomy. It may be added that Cruickshank (1913) has more recently failed to find that ingestion of fructose is followed by the deposition of glycogen.

After discussing various views as to the mechanism by which the pancreas prevents diabetes (such as the intoxication theory, the local action of the pancreas, and Lépine's glycolysis theory), Minkowski suggests as possible explanations : (1) That the conversion of glucose into glycogen is an essential preliminary step to its utilisation by the tissues, and that this requires " internal secretion " from the pancreas, acting either on the sugar itself or indirectly, through an influence on the cells of the liver or muscles. In order that glycogen formation from fructose may occur, the pancreatic influence, for some reason, is unnecessary ;

(2) that the pancreas acts either on the tissues in some manner which prepares them to form attachment with the sugar molecule, or on some loose compound, as which glucose is present in the circulating fluids, thus rendering it free, so that it becomes capable of being utilised by the tissues.

Excretion of acetone bodies was not a marked feature in Minkowski's observations. In three out of five cases β-oxybutyric acid (2-4 gms.) appeared in the urine only after the diabetes had persisted for two to three weeks, when the animals were very much emaciated and the sugar excretion was becoming much less than previously. Much more may have been produced in the organism, for it was found, on administering 10 gms. of sodium oxybutyrate to a depancreatised dog, that only 0·4 gm. reappeared in the urine, accompanied by much carbonate, so that the urine became alkaline. It is suggested, either that the oxybutyric acid may be a precursor in the synthesis of sugar out of protein, or that it arises (from protein) only when production of sugar from this source has become interfered with. The second possibility is considered by Minkowski as the more likely one. Complications such as peritonitis, necrosis of the duodenum, abscesses, etc., may be associated with a marked reduction in glycosuria.

In a paper appearing in 1908, Minkowski discusses the question as to whether or not the depancreatised animal has entirely lost the power of utilising glucose. The experiments of Seo and of Allard (1908) are referred to in this connection.

Seo had found that the excretion of sugar became less when a partially depancreatised dog was made do muscular exercise, whereas a completely depancreatised one showed very little change, unless in cases in which the resting D : N ratio was low, as a result of prolonged starvation or infection, when both the absolute amount of dextrose and the D : N ratio rose considerably following the exercise (D : N ratio might rise to 4·3). Minkowski concludes that there can be no increased utilisation of sugar in entire absence of the pancreas. Allard (1908) investigated the recovery of injected glucose. In one dog, which is described as having been completely depancreatised, although the D : N ratio was only about 1·0, more sugar reappeared in the urine than was injected, whereas in another in which some pancreas was left it did not all reappear. The results are therefore not very convincing.

Further studies of the effects of pancreatectomy were published by numerous other investigators, and we will refer here to those of Pflüger, Hédon, Lépine, Verzár, and Murlin.

Pflüger (1907), observing that separation of the pancreas from the duodenum in frogs was followed by glycosuria—although this did not happen if the blood-vessels between them were ligated—concluded that the anti-diabetic function of the pancreas is controlled by the nerve plexus in the duodenal walls. Attempts to resect the entire duodenum, in dogs, met with no success, although Weintraud had previously shown that removal of the lower portion of it (along with the rest of the small intestine) caused only temporary glycosuria. Ehrmann (1907) succeeded in keeping dogs alive up to a week after complete excision of the duodenum, but without observing more than a temporary post-operative glycosuria. Pflüger, for no very obvious reasons, would not accept this evidence as conclusive, so that Minkowski (1908) undertook to settle the question. He excised the duodenum and the head and body of the pancreas in one operation, leaving a portion of the latter either *in situ*, or as a graft under the skin. Only post-operative glycosuria was observed until, at a second operation, the remaining portion of pancreas was removed, when true diabetes followed. This disposes once and for all of Pflüger's idea that the presence of a nerve centre in the duodenum is necessary for the antidiabetic functioning of the pancreas.

Hédon (1910), in a general review of the internal secretion of the pancreas, points out that the graft experiments, as performed by Minkowski and von Mering and, simultaneously, by himself, do not entirely rule out of court the possibility that stimulation of sensory nerves in the pancreas itself might transmit, to the nerve centres controlling the glycogenic and the glycolytic functions of the various organs, impulses which maintain the balance of carbohydrate metabolism, as represented by the blood sugar level. It is considered possible that afferent impulses, acting on the glycogenic and glycolytic centres, may arise in several organs (Claude Bernard had suggested the lungs, for example). These arising in the pancreas are essential to maintain the tone of these centres and only a portion of this gland, left as a graft, is sufficient for this purpose.

Although, as Minkowski had also observed, severance of the original vascular connection between the graft and the duodenum is often followed by diabetic symptoms—because the newly

formed vessels between the graft and the tissues into which it was transplanted had not become sufficiently developed—Hédon found, in several animals, that this connection could be cut *without* any diabetes occurring. This rules out the possibility that afferent impulses travel by way of the pedicle, but it still leaves open the chance that they might be transmitted through nerve connections formed along with the new blood-vessels to the graft. In view of these possibilities, Hédon attempted to obtain irrefutable evidence as follows:—

He anastomosed the artery and vein of the freshly excised uncinate process of the pancreas of a large normal dog with the carotid artery and the jugular vein, respectively, of a small depancreatised one, without finding that there was any reduction in the glycosuria in the latter. He then collected 320 c.c. blood from the vein of the uncinate process through a paraffined cannula, allowed it to clot, and injected the 150 c.c. of serum which he obtained into the leg vein of a diabetic animal, but again without observing any reduction in the glycosuria. Since the supposed internal secretion might be destroyed, either during clotting or because of the time taken for collecting the blood, direct transfusion of fresh blood from a large normal dog into a small depancreatised one was performed, by establishing a crossed circulation between the carotid artery and the jugular vein of the two animals. The small animal (8 kg.) was placed on a balance and 375 c.c. of blood removed from it; it was then transfused until the original weight was regained. This was repeated three times. The blood sugar was originally 0·390 per cent., and after each transfusion it was 0·312, 0·243, and 0·240. Although the blood sugar was not brought down to the normal level, the glycosuria became very distinctly reduced, and Hédon concluded that the permeability of the kidney towards sugar must have become lessened. This may have been due to the hyperthermia and the albuminuria, which were noted after the transfusion. Finally, Hédon established a crossed circulation between the carotid arteries of a diabetic and a normal dog, and in the time during which this was maintained (up to ten hours) he found that the blood sugar of the two animals became nearly the same, between 0·2 and 0·3 per cent.—being somewhat higher in the diabetic animal—and the glycosuria of the diabetic animal diminished, accompanied usually by partial anuria. Slight glycosuria also developed in the normal animal. After disuniting the animals the glycosuria reappeared very rapidly in the depancreatised one.

Hédon sees in these results further evidence of a change occurring in the permeability of the kidney towards sugar, as a result of transfusion of the diabetic animal with normal blood, and he does not abandon the hypothesis that there may be a reflex nervous connection between the pancreas and the liver,

through which the glycogenic function of the latter is controlled. This he considers to be of a local character, since pancreatectomy still causes the usual degree of diabetes when it is performed on dogs in which the cervical spinal cord has been severed.

Lépine's [1] chief contributions to our knowledge of pancreatic diabetes were, that the so-called glycolytic power of the blood is less than normal, and that maltose may appear in it. He thought that the pancreas produces an internal secretion which favours glycolysis. Other observers have not succeeded in confirming these findings (see p. 194). He denied that the symptoms of diabetes are at all diminished by injections of suspensions of fresh pancreas, and states that transfusion of blood from a normal into a depancreatised animal only temporarily reduces the glycosuria, but has no effect on the hyperglycæmia.

Turning now to another aspect of the problem, namely, whether the depancreatised animal has entirely lost the power to oxidise sugar, the experiments of Macleod and Pearce (1913) and of Verzár (1913-14) may be referred to. Our interest in the problem was aroused by the conclusion which Knowlton and Starling (1912) at first drew from their experiments on the surviving mammalian heart (heart-lung preparation), namely, that decidedly less sugar disappeared from the perfusion fluid when the heart of a depancreatised animal was used than disappeared with one of a normal animal. We considered that if this difference was manifested by the heart alone, it should also be so when the other muscles were also included, a preparation of this type being obtained by ligating all the blood-vessels of the liver and digestive organs. The experiments, therefore, consisted in a comparison of the blood sugar at intervals after the above operation in normal and depancreatised dogs. As had previously been observed by Bock and Hoffmann, by Pavy (1903), and by Macleod (1909), the blood sugar gradually falls under these conditions. Although the rate of fall varies considerably in different animals, we found, on an average, that there was no difference in this regard between normal and depancreatised ones.

Thus the average consumption of glucose in observations

[1] In most of Lépine's observations pancreatectomy was performed after ligation of the pancreatico-duodenal vessels, so that the animals did not survive the operation for more than a few days because of gangrene of the duodenum.

on eleven non-diabetic animals was 1·63 mg. per minute, and that in ten diabetic ones, 1·86 mg. per minute.

Closely following these experiments, Verzár, in part along with Fejér, published researches in which the problem was attacked by measurements of the respiratory quotient (R.Q.). The rise in the quotient, which follows the intravenous injection of glucose in normal dogs, was found to persist until four days after removal of the pancreas, and from that time on glucose had no effect, indicating that the power to oxidise carbohydrate must have been lost. He considered that this result could not have been dependent on an inability of the organism to burn more sugar because of extra sugar being added to circulating fluids already containing more than could be consumed by the tissues. If such were the case, the quotient should not have risen following the injection of sugar on the second day, when, however, the blood sugar had already gained a very high level. It may be added that these observations were made on curarised dogs kept at constant temperature under artificial respiration, and that the same quantities of isotonic glucose solution per kilo body weight were injected in each case.

As judged by the same criteria, the power to oxidise fructose remained for much longer than was the case with glucose.

Since most authors agree that the glycogen stores become exhausted in from three to four days after pancreatectomy, it would appear that, for utilisation of sugar to occur, this substance must still be present in the liver.

Partial Pancreatectomy.—As has already been pointed out, considerable attention has been paid to the effect of partial pancreatectomy in dogs, because of the similarity of the condition thus established to diabetes as it occurs in man. When a certain proportion of the gland is left behind in the body, either as a graft, or, better still, in its normal position around the main duct, the animal may at first show no diabetic symptoms, even on a diet containing carbohydrates, but these gradually develop later, and become more and more severe until the animal ultimately dies. F. M. Allen (1913) has recently made extensive investigations of this form of experimental diabetes, and in his book has given an admirable summary of those of previous workers in the same field.

In a general way, it may be said that the particular problems

which lend themselves to investigation by this method are: (1) The structural changes which occur in the secreting cells of the remnant of pancreas left in the body (Homans, Allen, Bowie). Reference to this work will be found elsewhere in this volume (p. 25); (2) the influence of various conditions on the rate of development of severe diabetes. The importance from a practical standpoint of such knowledge need scarcely be emphasised, and the most important investigations have been contributed by Allen himself.

He has found, when about one-fourth of the pancreas is left (around the main duct which is not tied) that there is a demonstrable lowering of tolerance to sugar, as revealed by subcutaneous injections of sugar. This lowering of tolerance becomes more marked when only one-fifth of the gland is left, although the animal does not develop any diabetic symptoms, even when put on a carbohydrate-rich diet. When the remnant is one-sixth, a condition (diabetes levis) may supervene, in which the animal excretes sugar while on a starchy diet but not so on one of meat. With such a large remnant, however, the diabetic condition is usually only transient. Even one-tenth of the gland may suffice to protect the animal against diabetes, but usually the severe form (diabetes gravis) follows when from one-eighth to one-tenth is left. In this state glycosuria persists, even on a diet composed entirely of meat. No difference could be made out in the results according to whether the remaining portion of gland was a compact clump closely surrounding the main duct, or a long narrow strip.

A condition of diabetes levis may change to one of diabetes gravis, and it is obviously important to know the influences which may bring about the change. The most important of these is diet, and dogs in which severe digestive disturbance is avoided, by having the pancreatic remnant still attached to a patent duct, are much more suitable for studying its influence than is the case when, as in the graft experiments of Minkowski and Hédon, it is isolated from the intestine. The first observations in this direction were those of Thiroloix and Jacob (1912), who found that prolonged feeding of the partially depancreatised animals with carbohydrates led to permanent glycosuria, which did not disappear even when the animals were subsequently placed on a meat diet. Allen emphasises the fact, however, that all cases of diabetes levis do not thus become aggravated by carbohydrate feeding; some, on the contrary, may recover completely, although their sugar tolerance remains sub-normal. This would seem to indicate that the overstrain put on the

remaining portion of pancreas to produce the internal secretion necessary for carbohydrate metabolism leads to its breakdown. But it is extremely difficult to prove that this actually occurs. If it does so, it would be almost unique as an etiological factor, the only other instance in which degeneration from overstrain is known to occur being in an experiment by Barany, in which structural changes were observed to occur in the cells of the nucleus acousticus as a result of the receipt by them of persistent sound impressions. Allen does not unreservedly accept this hypothesis of overstrain, but he adopts it as a safe one to apply in the treatment of diabetes in man, and he sums up by saying: "Dogs which have lost a certain amount of pancreatic tissue will become diabetic irrespective of diet. Dogs which retain a sufficient amount of pancreatic tissue will never become diabetic irrespective of diet. But between these two groups is an intermediate group. On an Eskimo diet they may be found to live in health. On a Hindu diet they soon go down to fatal diabetes."

Prior to the work of Thiroloix and Jacob and of Allen, the effects of partial pancreatectomy were systematically investigated by Sandmeyer (1895).

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CHAPTER V.

THE HISTORY OF THE PREPARATION OF INSULIN.

The Earlier Attempts to Prepare Pancreatic Extracts having Anti-diabetic Effects.—Although one does not gather, from the earliest accounts of their investigations, that Minkowski and von Mering had in mind that the pancreas owes its anti-diabetic function to an internal secretion, this must have been the case, since in 1892 Minkowski published the results of experiments in which he had failed to observe any remedial action to follow injection into depancreatised dogs of extracts of the removed glands.

More encouraging results, however, had meanwhile been obtained by Capparelli, who found that in extracts of pancreas, prepared by means of isotonic saline, something was present which promptly reduced the glycosuria in depancreatised animals. During the next few years, until 1898, innumerable investigators, stimulated partly by Minkowski and von Mering's discovery, and partly by the knowledge that administration of thyroid gland removes the symptoms associated with deficient functioning of this gland, continued the search for the hypothetical anti-diabetic hormone. Many of these were clinical workers who did not, as a rule, venture to give the pancreas preparations otherwise than by mouth. In the light of our present knowledge, it is therefore not surprising that failure was the usual outcome of their investigations. Ausset, in 1895, noted improvement by giving raw pancreas to a depancreatised dog and also to a diabetic patient, and Bormann (cf. from Murlin) confirmed this, by giving subcutaneous injections of raw pancreas. But these favourable results were denied by Vanni and Burzagli, and so the accounts of the researches at this time read: a few of them recording doubtfully positive, but most of them entirely negative results.

Of the investigations appearing about 1898, those of Hougounenq and Doyon, of Hédon, of Lépine (loc. cit.) and of Blumenthal deserve some attention. The first of these workers prepared pancreatic extracts by many different methods, but found them entirely without effect when administered to depancreatised dogs by stomach, a result which was confirmed by Hédon, using glycerine extracts. Lépine and Martz found that the injection of lymph from a normal dog caused, when

injected intravenously, a certain degree of disappearance of sugar from the blood of rabbits, and they drew the conclusion that a glycolytic ferment must be present in it. But at this stage it was Blumenthal who came nearest to discovering insulin. He expressed juice from the pancreas by means of an hydraulic press, and then removed some of the proteins from it by alcohol. The extract, injected into laboratory animals and diabetic patients, gave evidence of increasing the utilisation of sugar (assimilation tests, p. 217), but it had such violent toxic effects that nothing came of the research.

It may be said that during the first decade of the present century the only serious attempts to treat diabetes by preparations of pancreas were those of Rennie and Fraser, Lépine, and Zuelzer. Rennie and Fraser used the principal islets of bony fishes (p. 31), and administered them usually by mouth, either in the raw state or as extracts, although in one case a saline emulsion was injected subcutaneously. The results were not conclusive.

In 1909 Lépine's book was published, and in it are described the researches from his laboratory bearing on the control of glycolysis in the body by means of an internal secretion derived from the pancreas. The attempts to apply pancreatic extracts in the treatment of diabetes are summed up in the sentence "mais les resultats n'ont nullement répondu aux espérances, ce qui peut s'expliquer a l'aide de diverses hypothèses."

By far the greatest advance was made by Zuelzer and his collaborators, who came very near to the discovery of a method for preparing insulin, as we know it to-day. With the object, apparently, of causing the internal secretion to accumulate in the gland, the pancreatic veins were ligated in recently fed calves about an hour prior to killing the animals, the pancreas was then excised, minced, extracted, and treated with alcohol, so as to yield an extract which was tolerably free of protein. This extract, after removal of the alcohol, was capable of diminishing the glycosuria resulting in dogs from injection of epinephrin, and, in one animal, from extirpation of the pancreas. Zuelzer proposed that the anti-diabetic strength of his extract, which was usually given intravenously, should be measured by seeing how much epinephrin it could antidote, as judged by the effects on blood sugar. He administered this extract, usually intravenously, to eight diabetic patients, and found not only that the glycosuria and the ketonuria became much less, or

disappeared, but also that improvement of the general condition of the patients was sometimes quite evident. The effects did not develop until the second or third day following the injections, but they lasted for several days. Unfortunately, however, the extracts were thought to contain some substance which caused toxic symptoms, including fever, and these symptoms were found by Forschbach, who tried them on patients in Minkowski's clinic, to be so alarming that, although the diabetic symptoms were alleviated, further use of the extracts in the treatment of diabetes was abandoned. Forschbach, however, reported favourable results when the extract was given to depancreatised dogs. The elusive pancreatic hormone came very near to being caught through Zuelzer's researches, and it is probable that "insulin" would have been really "bottled up" at this time had more attention been paid to a study of the effects of the extracts on laboratory animals, rather than diabetic patients. The toxic symptoms exhibited by the latter, although they are described as having been accompanied by fever, may very likely have been due to hypoglycæmia, and it seems certain, in the light of what we now know, that more intensive prosecution of the laboratory investigations, by revealing their true nature, would have removed the fear with which the symptoms were regarded in patients.

For some time following Zuelzer's important researches, belief that an extract containing the anti-diabetic pancreatic hormone was shared by but few. Leschke published negative results, and even challenged the idea of there being an internal secretion of the pancreas. He showed that the digestive enzymes of the pancreas have a highly toxic action on animals, and this he tried to prevent by heating his pancreatic extracts, but by so doing he apparently also destroyed the anti-diabetic hormone.

The next important attempt was that of E. L. Scott, who set out with the idea, previously suggested by Leschke and hinted at by Cohnheim, that the external (digestive) enzymes may destroy the anti-diabetic hormone of the pancreas. He therefore took precautions to prevent the effect of these enzymes. "It was hoped that the presence of the digestive enzymes could be eliminated by the atrophy of the gland which follows complete ligation of the ducts." Finding after several attempts that atrophy of the gland was incomplete, he changed the plan of the

research, and " in subsequent work these enzymes were rendered inactive at once by a high percentage of alcohol, and were later killed by long-continued contact with strong alcohol." The pancreas was finely macerated in a mortar, and extracted at about 40° C. with alcohol, enough of this being added to bring the percentage to 85. The extract was then evaporated at reduced pressure, and the residue, after extraction with ether, dissolved in 95 per cent. alcohol, again evaporated, and the residue dissolved in 0·85 per cent. NaCl solution. On injection, this extract gave no evidence of anti-diabetic properties. In the light of the more recent work it is likely that the active principle was lost, either when the pancreas was being macerated, or by its being precipitated by the 85 per cent. alcohol. More success attended further trials, in which alcohol-soaked pancreas was evaporated to dryness *in vacuo*, the residue washed in absolute alcohol, and then extracted with acidified water. After drying this extract it was kept for use under absolute alcohol. Injected into depancreatised dogs, solutions of the extract caused the urinary sugar to fall, in one animal, from 17·2 on the day on which the injection was made, to 8·9 on the day following, the D : N ratio meanwhile changing from 2·53 preceding the injection, to 1·93 following it. A certain improvement in the general condition of three out of four injected animals is also reported. The author was evidently satisfied that the extract lowered the urinary sugar, but adds : " It does not follow that these effects are due to the internal secretion of the pancreas in the extract."

In 1913 F. M. Allen summed up the work on the use of pancreatic extracts in the words, " All authorities are agreed upon the failure of pancreatic opotherapy in diabetes." This worker found, like Leschke, that extracts of various organs, made with weak glycerine, cause, when injected subcutaneously into normal animals, varying degrees of glycosuria, extracts from the pancreas having no less an effect in this direction than those from other organs. Influenced by these findings, Allen, in reviewing the literature, pays much more attention to the negative than to the positive results of other workers.

We see, then, that through nearly a quarter of a century following the announcement of von Mering and Minkowski's discovery (from 1889 to 1913), the attempts to prepare pancreatic extracts containing the anti-diabetic hormone, although without satisfactory outcome in the practical sense, were nevertheless not complete failures. Enough of success was met with to keep

alive the spark of hope that some day the elusive hormone would be run to earth, and this hope was greatly fortified by the coincident researches of the anatomists and experimentalists referred to elsewhere (p. 2). Particularly to Laguesse, Diamare, and Schafer do we owe much for their firm stand in favour of the view that the islets of Langerhans are the organs upon which the anti-diabetic function of the pancreas primarily depends. Hédon, among the experimentalists, through transfusion experiments, supplied the most weighty evidence for the existence of an internal secretion.

The next epoch in this history opens in 1913 with the researches of Knowlton and Starling, who, by using heart-lung preparations, thought at first that less sugar disappeared from the perfusion fluid when the heart was taken from a diabetic (depancreatised) animal than from a normal one, and that the sugar consumption could be increased in the former by adding a pancreatic extract (made by the same method as that used for secretin) to the perfusion fluid. Somewhat similar results were obtained by Maclean and Smedley. Macleod and Pearce could not, however, detect any difference in the rate at which sugar disappears from the blood of eviscerated dogs, according to whether normal or depancreatised animals were used; and Patterson and Starling subsequently published evidence to show that the results of Knowlton and Starling were confusing, partly because of leakage of fluid containing sugar into the lungs, and partly because of uncertainty as to the behaviour of the glycogen of the heart. Since no difference in the rate of sugar consumption in diabetic and non-diabetic hearts could be demonstrated with certainty, it was useless to try further the effect of pancreatic extracts. Unconvincing though these researches may have been, they must, nevertheless, be considered as of great value, because they pointed a new way to the investigation of the problem of sugar consumption by the tissues. They aroused new interest in the whole subject, and in the next few years appeared papers by Murlin and his collaborators, by Clark, and by Meltzer and Kleiner.

As a direct continuation of the heart experiments, we may first consider those of Clark, who, in 1916, reported that when Locke's solution (containing glucose in physiological concentration) was repeatedly perfused through the blood-vessels of the

pancreas of the dog (with strict aseptic precautions), and then through the excised beating heart, sugar utilisation became distinctly more rapid than that occurring when freshly prepared Locke's solution containing the same percentage of glucose was perfused. It was concluded that the pancreas supplies something to the Locke's solution which accelerates the utilisation of sugar by the living heart. Certain experiments suggested that this pancreatic influence must be due to the production of a substance possessing some of the characteristics of an enzyme, rather than of a stable internal secretion. Evidence was also obtained to show that the decrease of sugar, after perfusion of the heart, depends partly on its condensation to some non-reducing form, and partly to its destruction by hydrolysis or oxidation. In a paper published a year later, 1917, the further significant fact was revealed that although the reducing power was not changed after pancreatic perfusion alone, a distinct diminution occurred in the optical rotation of the perfusate, indicating that the pancreas exerts an independent influence on glucose. This change in optical activity could also be brought about when glucose was added to a glucose-free Locke's solution, after this had been perfused through the pancreas, and the mixture incubated. In both cases, osazones with lower melting-points than that of glucosazone were prepared from the perfusates. Not only the reducing power, but also the optical rotation and the melting-point of the osazones, were raised by hydrolysing the solution perfused through both pancreas and heart. It is significant that with fructose similar results were not obtained.

These experimental results were considered by the author to "suggest that the enzyme or enzymes derived from the perfused pancreas have a specific action on dextrose, and are responsible for certain essential steps by which dextrose is prepared for normal utilisation." There is no doubt that they are highly suggestive, although several of the values for changes in rotatory power which are recorded are not very large, and may, we think, have been due to the influence of traces of protein in the solutions.

Between 1913 and 1916 Murlin and his associates continued the search for the pancreatic hormone. In a review of this work, published by Murlin, Kramer, and Sweet, it is stated that the first observation was one in which the injection of a concentrated

saline extract of ox pancreas caused complete disappearance of sugar from the urine of a diabetic (depancreatised) dog. In subsequent experiments the extracts were made alkaline before injecting them, and it was thought that the decline in the excretion of sugar which followed might have been due to the alkali alone, a view which was apparently confirmed by finding that injection of an amount of alkaline Ringer's solution equal to that of the pancreatic extract "produced a decline in the sugar excretion to the same hourly level, and the same G : N ratio." "This singular coincidence diverted our attention for several years to the alkali, although we were convinced in 1916 that the pancreatic extract played a part in the various signs of improvement reported." The power of diabetic animals to oxidise carbohydrate had also been shown in 1913 to be somewhat increased by the injection of pancreatic extracts, and in 1916 a more decided effect was obtained, by injecting a suspension of finely ground pancreas in Ringer's solution. These authors also had the idea that "secretion of some constituent of the (duodenal) mucosa might be an activating agent for the internal, as well as for the external, secreting mechanism"; but this proved not to be a fruitful idea. Because of military duties, Murlin and his collaborators were compelled to abandon further prosecution of the research, and they were unable to take it up again until after Banting and Best's experiments had become known, and Collip had succeeded in purifying the extract sufficiently for repeated injection into patients. With Zuelzer, E. L. Scott, and Rennie and Fraser, Murlin and his collaborators came nearest to demonstrating that insulin can be extracted from the pancreas, and had too much regard not been taken of what were thought to be possible sources of error, it is possible that insulin might have been available for the treatment of diabetes ten years before this was actually the case.

Kleiner and Meltzer, and later Kleiner, showed that the slow intravenous injection of a suspension of pancreas in weak saline can diminish the degree and the duration of hyperglycæmia in depancreatised dogs given sugar. Suitable controls showed that these effects were not dependent on dilution of the blood by the injections, and Kleiner suggested that an alteration in the permeability of the capillary wall towards glucose might be a factor in the result.

The Isolation of Insulin.—The researches which have just been reviewed gave strong support to the belief that insulin is present in the pancreas, and the problem was to devise means by which it could be extracted in a condition suitable for repeated

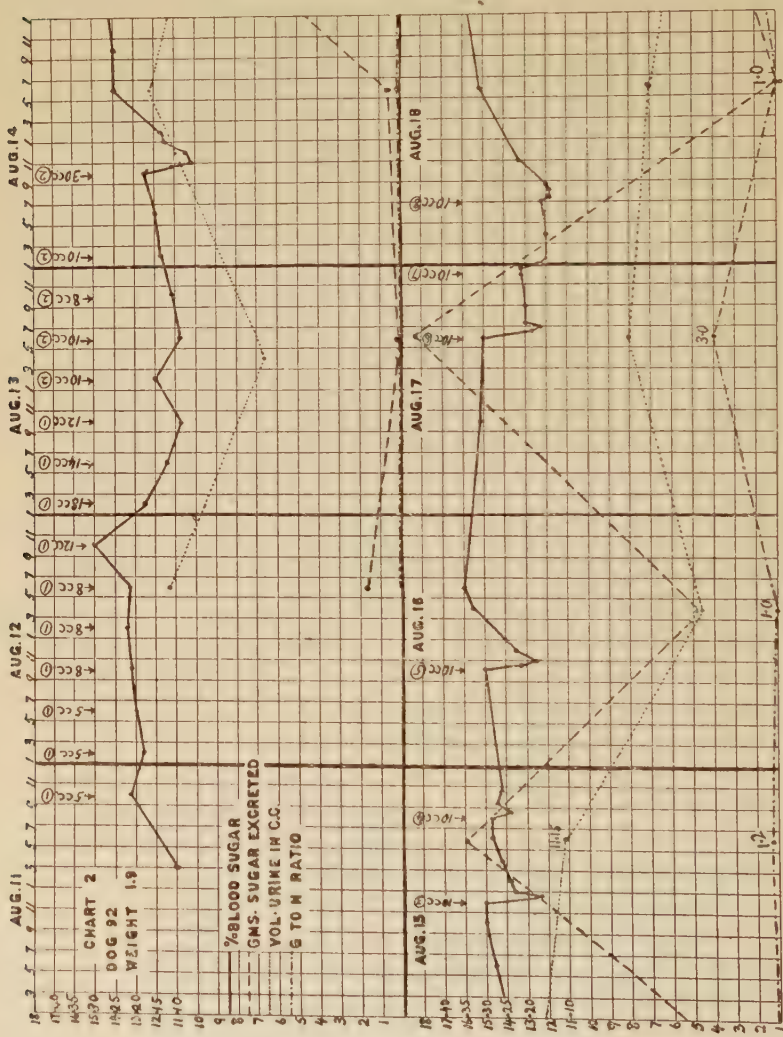

FIG. 10.—Chart of one of Banting and Best's experiments.

injection into man. With the object of preparing such an extract, F. G. Banting, in 1921, (1) undertook in my laboratory to reinvestigate the problem, and suggested, as a first step, using duct-ligated pancreas, so as to circumvent the destructive action of trypsin. After full consideration of the methods to be adopted

in testing the efficiency of the extracts, it was decided that the blood sugar and the urinary sugar in diabetic (depancreatised) dogs should be observed at frequent intervals, before and after their parenteral injection. To make the carrying out of this

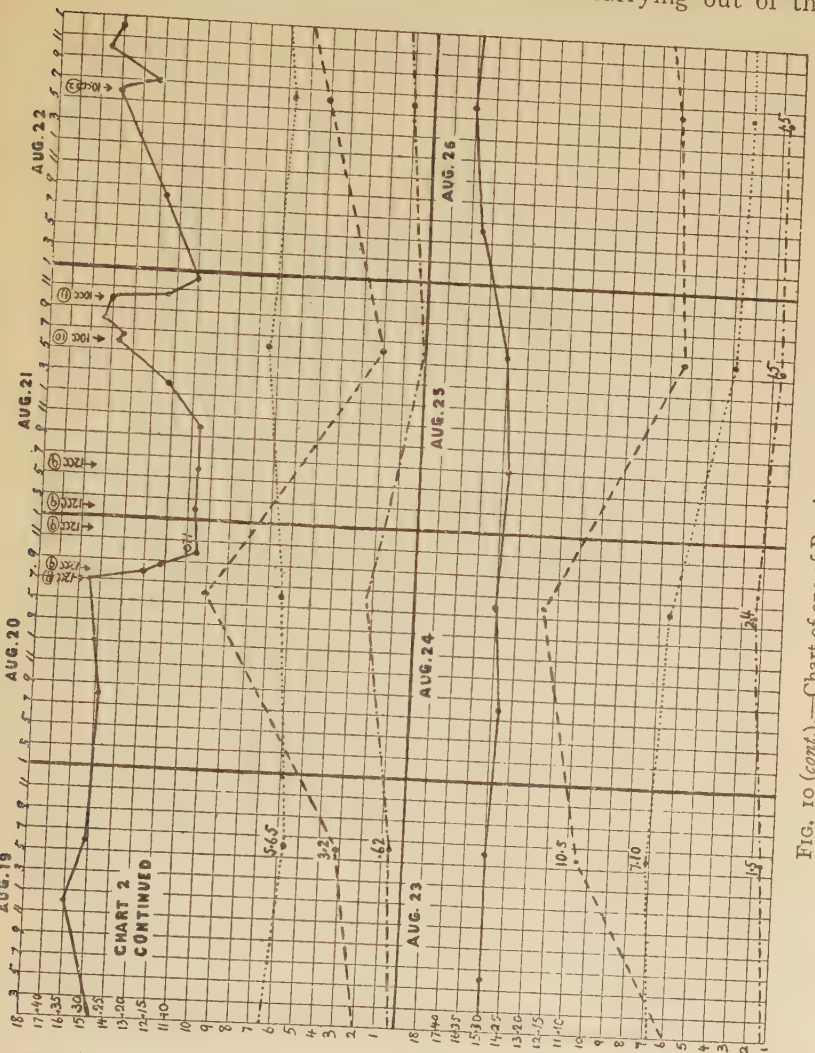

FIG. 10 (*cont.*).—Chart of one of Banting and Best's experiments.

exacting programme possible, C. H. Best took part in the work. As a first step, the pancreatic ducts were tied in several dogs, with precautions to see that no secretory pathway still remained patent (see p. 19). Results of a typical experiment are shown in Fig. 10. The animal was depancreatised on 11th August, and six

hours after the operation an extract, made by means of ice-cold Ringer's solution from the residue of pancreas remaining ten weeks after ligation of the ducts, was injected. This apparently retarded the increase in blood sugar, since it scarcely rose above 0·2 per cent. until 10 P.M. on 12th August, by which time it would otherwise have been decidedly higher (p. 44). Because of the increase which did occur at this time, a larger amount of extract was injected, with the result that the blood sugar next day declined to 0·10 per cent. At this time the supply of the original extract was all exhausted, so that one from the degenerated gland of another animal had to be used, with the result that moderate quantities of it succeeded in holding the blood sugar below 0·20 per cent. On 14th August the usual dose of this extract was greatly increased, and the blood sugar fell from 0·175 per cent. to nearly 0·09 per cent. Up to this time there had been very little sugar in the urine, but the extracts being now discontinued this quickly rose, so that, on 15th August, 16 gms. were excreted in the twenty-four hours; meanwhile, also, the blood sugar had been steadily rising again. Further injection of extract from degenerated pancreas caused prompt reduction in blood sugar, as well as in urinary sugar.

It was clear from these results that the severity of the diabetic symptoms could be distinctly diminished by the extract of degenerated pancreas, and the available supply of this being exhausted, extract from the normal gland was injected, on 17th and 18th August, when, as will be seen from the chart, results similar to those observed with the degenerated extract were obtained. Extracts prepared from the pancreas of a dog, after repeated injections of secretin, were likewise successful. After 22nd August the injection of pancreatic extracts was discontinued, with the result that the diabetic symptoms became pronounced, the blood sugar rising to between 0·30 and 0·35 per cent., and the daily urinary sugar excretion, to between 6 and 13 gms. The animal died on 30th August, and no trace of pancreas could be found by naked-eye examination at autopsy.

Experiments of a similar type were made on nine other depancreatised dogs, with entirely confirmatory results. In several of them extracts prepared from other tissues than the pancreas were found to have no anti-diabetic effects. Administration *per rectum* was without action. Attempts were also

made to measure the increased disappearance of glucose, as the result of injection of the extracts, by seeing what proportion of an amount injected intravenously was recoverable from the urine, with and without insulin. Thus, in one experiment without insulin, the excretion of glucose during four hours following the injection of 10 gms. was 9·94 gms., whereas on the next day, when extract was also given, it was only 4·4 gms. It may also be mentioned that care was taken to see that changes in blood sugar were not due to dilution of the blood (hæmoglobin estimations).

In view of the large amount of work which had previously been done in this field, it was considered advisable to make certain of the anti-diabetic effects of the extracts, as judged by the behaviour of blood sugar and urinary sugar, before proceeding to investigate their influence on other symptoms of diabetes, such as glycogen formation, ketosis, and changes in respiratory metabolism. The difficulty at this stage was to obtain adequate supplies of extract, so that Banting and Best devoted their attention mainly to this problem. With this object in view, (2) they made use of the fœtal ox pancreas, since it had been shown by Ibrahim that up to four months the acini are not sufficiently developed to secrete active trypsin, although the islets are abundant. Pancreases from fœtal calves were therefore extracted, either with Ringer's solution or with alcohol, which was then removed from the extract by evaporation in a current of warmed air, and the residue redissolved in Ringer's solution. Decided anti-diabetic effects could readily be demonstrated by these extracts, and this suggested the attempt to make them from the pancreas of full-grown cattle, by extraction with equal volumes of 95 per cent. alcohol made slightly acid with HCl. This extractive was used with the object of minimising the destructive action of the proteolytic enzymes, and its possible value in the preparation of insulin, previously suggested by Zuelzer and Scott, had been in mind from the very start of the investigations. After the removal of the alcohol, by warmed air, and of excess of fat, by toluene, the extracts were found to possess strong anti-diabetic properties, and it was now possible to show, beyond doubt, that by continuous injections, a great improvement occurred in the general condition of the animals, one of which lived for seventy days, when it was killed by chloroform.

On gross examination, no trace of the pancreas could be found, but serial sections of the duodenum, made by Dr. W. L. Robinson, revealed the presence in the submucous coat, near the entry of the main pancreatic duct, of a small nodule in which, however, no islets could be seen.

An endeavour was now made to purify the alcoholic extracts of adult pancreas sufficiently for trial on diabetic patients. The alcohol was removed by warmed air, or *in vacuo* at a low temperature, the excess of fat extracted by toluene, and the watery residue, now reduced to one-fifth the original volume, was passed through a Berkefeld filter. The resulting extract, injected into a diabetic patient (a boy aged fourteen years), lowered the blood sugar by a little over 25 per cent., and somewhat diminished the glycosuria, but "owing to the high percentage of protein . . . sterile abscesses formed in a few instances at the site of injection." Banting and Best (3) stated that the potency of their extracts was destroyed by heat and by digestion with trypsin, and that the active principle was insoluble in 95 per cent. alcohol.

Before further attempts could be made to investigate the possible therapeutic value of the extracts, it was necessary to remove from them the irritating substances responsible for abscess formation, and to demonstrate, in diabetic dogs, not only that the hyperglycæmia and glycosuria are reduced by their action, but also that the other diabetic symptoms are removed. At the same time, it was considered important to see whether other forms of experimental hyperglycæmia, such as that caused in rabbits by piqûre or epinephrin, would be affected by the extracts.

The problem of purification was entrusted to J. B. Collip, who, as a first step in his work, injected some of the crude extract into normal rabbits, and found the blood sugar to become reduced. This furnished him with a method for testing the potency of the various precipitates and filtrates which were produced in the crude alcoholic extracts by various strengths of alcohol. He finally found that the active principle remained in solution up to an alcohol percentage of about 92, and that, by using percentages somewhat below this, much of the protein could be precipitated from the extracts.

While this work was in progress, in Toronto, a paper by

Paulesco came to our notice, and after it was completed, one by Gley.

Paulesco's researches were communicated at a meeting of the Reunion Roumaine de Biologie in the spring of 1921, when he described the effects produced by intravenous injections of sterile pancreatic extracts on the percentage of sugar, of acetone bodies, and of urea in the blood and urine of depancreatised dogs. Typical observations are shown in Table II. :—

TABLE II.

Pancreatectomy.	Injection.	Blood.		Urine.	
		Glucose per 1000 c.c.	Urea gr.	Urea per 1000 c.c.	Glucose gr.
Before		0·96	0·50	15·00	0·00
After	Before	1·56	1·20	44·00	24·20
	After 1 hr.	0·90	0·95	14·00	0·00
	After 2 hrs.	0 62	0·90	26·00	0·00
	After 16 hrs.	1·48	1·20	49·00	abundant
	After 48 hrs.	2·00	1·80	—	abundant

Pancreatectomy.	Injection.	Glucose per 1000 c.c.	Acetone gr.	Acetone per 1000 c.c.	Glucose gr.
Before	—	0·88	—	0·008	0·000
After	Before	1·22	0·027	0·019	18·70
	After 2 hrs.	0·32	0·016	0·012	14·40
	After 24 hrs.	1·66	0·022	0·033	6·60

There can be no doubt that all three substances became markedly reduced in amount, in both blood and urine, as a result of the injections. The results were the same whether the injection was made into a branch of the portal vein or into the jugular vein. The effects were noticeable in one hour following the injection, attained their maximum in two hours, and passed off in twelve hours. They varied with the amount of gland present in the injected extract. Paulesco also observed that the blood sugar, as well as the blood urea, in a normal dog became lowered by the injections. It is evident that some error must have been incurred in the measurement of the blood sugars, the value for normal dog blood being given as 0·044 per cent., and for the same animal, two hours after the injection, as 0·028 per cent. At such percentages violent hypoglycæmic symptoms would have been manifest. The highest blood sugar recorded

after pancreatectomy is 0·27 per cent. No observations are recorded of the behaviour of the respiratory quotient or of the glycogen content of the liver, and no evidence is given that the general symptoms of diabetes were lessened, or the life of the animal prolonged.

At the meeting of the Société de Biologie, held in Paris on 23rd December, 1922, to commemorate the centenary of the birth of Pasteur, Professor E. Gley requested that an envelope, deposited by him in February, 1905, be opened and read. In this communication, after referring to his earlier researches, in which it was shown that destruction of the pancreas *in situ*, as by the injection of foreign materials into the ducts, does not lead to diabetes, Gley states that it is probable, as indicated by the work of Laguesse, that this was because the islets of Langerhans remained intact. He suggests that the failures of previous investigators to benefit the symptoms of diabetes, by injecting extracts of the entire gland, may have been due to the presence of other substances besides the active principle of the islets. He therefore prepared extracts from sclerosed remains of pancreas, and found them to diminish considerably the sugar in the urine of completely depancreatised dogs, and to alleviate all the other diabetic symptoms. Gley then indicates his intention to isolate the active anti-diabetic principle, to study its mode of action, and to see whether the extracts could be used on man, either subcutaneously or by mouth. Because of other researches, these problems were laid aside. Previous to depositing this sealed package, Gley had contributed valuable observations concerning the effects on depancreatised dogs of extract of the entire pancreas prepared in various ways, and also of defibrinated blood collected from the pancreatic vein. It was as a consequence of the negative results obtained by these methods that he proceeded to use degenerated glands, as he stated he had done when he deposited the sealed package in 1906.

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CHAPTER VI.

THE PREPARATION AND CHEMICAL PROPERTIES OF INSULIN.

Since a method suitable for the manufacture of insulin on a large scale was first described, numerous modifications have appeared, some of them involving principles essentially different from those upon which the original method of Collip depends. Without entering into details with regard to the various problems of chemical technique and engineering which are involved, a brief outline of the more important large-scale methods may be of interest. An excellent detailed description of the published methods will be found in the article by Grevenstuk and Laqueur.

The Method Elaborated by Collip.—

Freshly minced pancreas is allowed to stand, with occasional stirring, for a few hours with about an equal volume of 95 per cent. ethyl alcohol, after which it is strained through cheese cloth and the extract filtered through paper. Sufficient alcohol is then added to the filtrate to bring the percentage of alcohol to about 60, when, on standing, the major part of the protein separates out, and is removed by filtration. This second filtrate is concentrated to small bulk, by distillation *in vacuo* at a low temperature, and fat and other lipoid substances are removed, partly by skimming and partly by extraction with ether in the separating funnel. The purified extract is returned to the vacuum still and concentrated until of a pasty consistency, when alcohol is added so as to give a total percentage of 80. The mixture is centrifuged, whereby an upper layer consisting of alcohol containing the active principle in solution separates out. This is pipetted off and the insulin precipitated by throwing it into several volumes of absolute alcohol. After standing some hours this precipitate is collected on a Buchner funnel, dissolved in distilled water, and the solution passed through a Berkefeld filter. Although the resulting insulin is sufficiently free of impurities so that it can be used for repeated clinical administration it is, nevertheless, coloured and contains a considerable percentage

of inorganic salts and of protein.[1] Various methods have, therefore, been suggested for its further purification, the best known being those of Doisy, Somogyi, and Shaffer, and of Dudley.

The Method of Doisy, Somogyi, and Shaffer.—

The alcohol with which the initial extractions are made contains 20-30 c.c. con . H_2SO_4 for each kilogram of pancreas, and the first extraction, after removal of the alcohol in the vacuum still or in a current of warmed air, is mixed in a separating funnel with ammonium sulphate in the proportion of 40 grams to each 100 c.c. of solution. After standing at a low temperature, a precipitate rises to the surface and sticks to the walls of the funnel. Since this contains the major part of the insulin, the liquid under it is drained off, the precipitate dissolved in water, reprecipitated with $(NH_4)_2SO_4$, and finally dissolved in water containing sufficient ammonia to bring pH to between 6 and 8. By centrifuging, a clear watery extract is obtained, to which weak acetic acid is added, so as to bring pH to about 5. After standing at a low temperature for some hours, the precipitate which forms is collected and redissolved in water containing sufficient acid (HCl) to bring it into solution. The insulin is then reprecipitated by readjusting the pH to between 5 and 6, and the final precipitate collected on a filter and dried in a vacuum desiccator. From this dried material insulin of any desired strength can then be prepared by dissolving in weak acid.

The Method of Dudley.—

Insulin, prepared by Collip's process, is dissolved in a small quantity of water and the solution centrifuged, so as to free it from insoluble material. The supernatant fluid is then diluted with water to bring the concentration of the original crude insulin to about 1·5 per cent. pH adjusted to about 5, half the volume of a saturated watery solution of picric acid added, and the mixture allowed to stand some days in a tall vessel. By this time a yellow precipitate settles out (insulin picrate), and the supernatant fluid is removed by decantation, the precipitate being dissolved at a low temperature in a minimum of water containing weak sodium carbonate. From this solution the insulin picrate is reprecipitated by neutralising with acid, some more saturated picric acid solution being added to ensure complete precipitation. After standing some days this second precipitate is collected on a Buchner funnel and thoroughly washed with weak picric acid solution, then transferred to a beaker and stirred with a solution of HCl in 75 per cent. alcohol. The correct proportion of HCl is obtained by

[1] It was found advantageous, when trying to develop this method on a large scale, to use 95 per cent. acetone in place of alcohol for the first extraction and to make this faintly acid by means of acetic acid (0·1 per cent.). Evaporation in a warmed air current was used instead of the vacuum still, one advantage being that the fats separated out readily. The concentrated extract (about one-tenth the original volume) was then treated with alcohol, as recommended by Collip (cf. Best and Scott).

taking 25 c.c. of 3 *N* HCl in 75 c.c. absolute alcohol. On mixing the acid alcohol with the picrate thick, dark brown, oily drops are first formed. These afterwards dissolve, by stirring, in the acid alcohol, to form a slightly turbid, yellow liquid. By the addition of about ten volumes of pure acetone, insulin hydrochloride precipitates out from this solution and is collected on a filter, washed with acetone, and finally with ether, until all traces of picric acid are removed. After drying in a vacuum desiccator, a white powder of tolerably constant composition and strength is obtained. It should be kept in a sealed tube, or over P_2O_5 in a desiccator.

A few of the modifications of these original methods may be alluded to. Krogh and Hagedorn have considerably increased the yield of insulin obtainable from ox pancreas by freezing the freshly removed glands. The blocks of ice are then cut by rapidly revolving knives into very thin shavings which are collected in acidified alcohol (pH_2). The reaction of the alcoholic extract is then readjusted to pH 4·6 by means of lime, and after reduction in volume the insulin is purified by means of $(NH_4)_2SO_4$. The solution of crude insulin is boiled for two minutes at a pH below the isoelectric point, so that Berkefelding is dispensed with.

Brailsford Robertson, and Anderson have considerably cut down the amount of alcohol required in the original process by using exsiccated sodium sulphate. They add to the first 50 per cent. alcoholic extract of the pancreas sufficient sulphate to remove four-fifths of the water present, thereby raising the percentage of alcohol in the mixture to about 80, at which concentration protein fractions not containing insulin are precipitated.

Several papers have appeared from time to time by Murlin and his co-workers describing various methods for the preparation of insulin. The most approved method consists in preserving the pancreas in chilled 0·2 per cent. HCl and then, after mincing, heating it (to 75° C.) in 4 volumes of acid of this strength for an hour. After chilling the fat is skimmed off and the mixture is strained through cheese cloth. The pH is then adjusted to 4·9, the solution filtered through coarse filter paper, and 250 gms. NaCL is added to each 1000 c.c. of filtrate. The precipitate which settles out contains all of the insulin, as well as some protein. The insulin is dissolved out by 70 per cent. alcohol, and the alcoholic extract shaken with 3-5 volumes of amyl alcohol and centrifuged. A precipitate forms between the aqueous and alcoholic layers. It is dissolved in 80 per cent. alcohol, filtered, and the alcohol removed by evaporation *in vacuo*. A watery sterilised solution of the residue is insulin. For the preparation of a form of insulin which gives no biuret test the authors recommend using perfusates obtained by circulating 0·2 per cent. HCl through the blood-vessels of the excised pancreas. The pH of the perfusate is adjusted to 5·85, at which metaprotein is thrown down. After filtering, pH is adjusted to 4·1 and NaCl added (1 gm. to 3·5 gms. pancreas), after which the fluid is evaporated to dryness and the residue repeatedly treated with 80 per

cent. alcohol. After removal of the alcohol by evaporation the residue is treated with sterile water and pH adjusted to 4·1.

Moloney and Findlay attained considerable success in the purification of insulin by taking advantage of the fact that it is adsorbed by benzoic acid when this is caused to separate out by adding mineral acid (HCl) to a mixture of crude insulin solution and sodium benzoate. These workers have also made detailed studies of the extent to which insulin is adsorbed by other agents, such as charcoal, but the methods are not economically applicable on a large scale.

Through the work of Best and Scott and their collaborators in the Connaught Laboratories of the University of Toronto, and of Clowes, Walden and others in the laboratories of Eli Lilly and Company of Indianapolis, numerous details of practical value have been elaborated and applied in the manufacture of insulin.

The Method of Dodds and Dickens.—

This is a modification of the method of Dudley, with the difference that picric acid is directly mixed with the pancreas, instead of with crude insulin as prepared by Collip's process. As a matter of fact, the use of picric acid as the first step had been suggested by Dudley for the preparation of insulin from the principal islets of fishes prior to its use by Dodds and Dickens for mammalian pancreas. The process is relatively simple and should be less costly than the older ones. The chilled, fat-trimmed pancreas is passed through a mincing machine along with finely powdered picric acid and the process repeated several times. The picric acid unites with insulin to form the picrate, which is then leached out from the yellow paste by the addition of sufficient acetone to give a concentration of 70 in the mixture, and thorough trituration. The acetone extract is filtered through cloth under a press and extraction repeated several times with 70 per cent. acetone. The acetone is removed from the clear combined extracts by evaporation *in vacuo*, and the mass of picrate, fats, and picric acid crystals which remains is transferred to a Buchner funnel on which it is rubbed up with ether, which is then sucked through, this process being repeated until all fats and picric acid have been removed. The picrate is then converted into hydrochloride by the Dudley process (p. 71).

As a method for the preparation of insulin from the principal islets of fishes, this method, as originally used by Dudley, is very satisfactory, especially since the islets can very conveniently be preserved in moistened picric acid, in place of alcohol. The only precaution to observe is that the islets should be crushed and stirred in among the picric acid crystals, otherwise sufficient penetration does not occur to prevent deterioration. We found this to our cost in a large amount of islets removed from halibut (*Pseudopleuronectidæ*). The islets, which are large and very accessible in this fish, were merely dropped into a saturated solution of picric acid, but in several weeks' time, when they reached the laboratory, they were found to have decomposed, so that only traces of insulin were obtained.

The Chemical Properties of Insulin.—The majority of investigators consider insulin to be closely related to proteose (Dudley, 1923). In a general way, it is true that it closely resembles this protein in most of its reactions, but in many of them so faintly so that the possibility remains that insulin is a non-protein substance which for some reason remains attached to proteose, even after such chemical treatment as would be expected to rend them apart. It is really impossible to say what insulin is, for there is no reason to believe that it has as yet been isolated, even in a tolerably pure state, and until this is accomplished, it can be of little interest to review the numerous chemical properties which have been ascribed to it.

In what is probably the purest form, as prepared by isoelectric precipitation, insulin exists as a white powder, of which from 0·015-0·025 mg. corresponds to one unit (p. 242), and it contains 13-17 per cent. of nitrogen, no phosphorus, but considerable sulphur. The activity is often greater in preparations containing the least nitrogen (Mattill, Piper, Kimball, and Murlin). It dissolves with difficulty in strictly pure water, but readily so in a trace of acid or alkali. It is precipitated within a certain range of pH, the exact limits depending on the purity of the preparation, as well as on the nature of the solvent and the presence of electrolytes. In pure water it precipitates in the presence of strong acid, remains in solution between pH 2 and 4, precipitates again between pH 4·3 and 5·7, and remains in solution beyond pH 6 (Doisy, Somogyi, and Shaffer).

Insulin prepared from the pancreas of the skate, by the relatively simple process of extraction with about 60 per cent. acidified alcohol, and subsequent heating of the alcohol-free extract, failed to show any precipitation by adjustment of the reaction (Best and Macleod). In the presence of small concentrations of salts, especially sulphates, the isoelectric point shifts towards the acid side, and precipitation may occur at pH 4, or even less, and when much salt is present, such as $\frac{1}{3}$ to $\frac{1}{2}$ saturation with $(NH_4)SO_4$, or saturation with Na_2SO_4 or NaCl, insulin may become precipitated well below this level of pH. A multitude of other salts also precipitate insulin, as well as such reagents as picric acid, trichloracetic acid, etc. (cf. Widmark).

Insulin is soluble in ethyl alcohol up to a concentration of 80 per cent. provided the reaction is outside the isoelectric range, this being the

basic fact upon which Collip developed his method of purification. At this concentration of alcohol, most protein substances are precipitated, and trypsin fails to develop its digestive properties. It is said that within the isoelectric range insulin is more soluble in weak alcohol than in water (Grevenstuk and Laqueur). Insulin is also soluble in methyl alcohol and in glacial acetic acid, phenol, formamid, and the kresols. It is insoluble in alcohol above about 90 per cent. and in the fat solvents. In this regard, however, it should be stated that insulin prepared from fish pancreas (skate) gave no precipitate, even when a watery solution was dropped into absolute alcohol. With regard to other physical properties, the apparently purest preparations of insulin have all been found to be readily adsorbed, especially in acid solution, by kaolin, charcoal, benzoic acid, and Lloyd's reagent. This made it difficult, in the earlier stages of its manufacture, to avoid serious loss of insulin while sterilising the solutions by passing them through the Berkefeld filter, but Dudley has shown that this loss can be entirely prevented by adjustment of the reaction to pH 7·5. It is also possible to decolorise insulin solutions by means of charcoal provided the reaction be properly adjusted (Krogh and Hagedorn, private communication). Moloney and Findlay have made a careful study of the adsorption properties of insulin, and have suggested a method of purification based on them.

In numerous attempts to dialyse insulin through parchment or collodion sacs, no trace was ever found by us to pass out, but more recently Shonle and Waldo have succeeded. Dingemasse (cf. Grevenstuk and Laqueur) has been unsuccessful in demonstrating any dialysis of insulin. He attempted, by this method and also by that of electrodialysis, to separate insulin from traces of protein.

Contrary to the earlier findings of Banting and Best (p. 66), insulin readily withstands heat, at least when in faintly acid solution (pH4 or less). We have, for example, kept a faintly acid solution of insulin actively boiling under a reflux condenser for two hours without being able to detect any deterioration in potency. At reactions above pH5, however, insulin is destroyed by heat, the more rapidly the more alkaline the solution. Properly prepared solutions can withstand moderate temperatures, such as would be met with in the tropics, without any loss of potency, although in some preparations sent to the testing laboratories of the Insulin Committee, a certain cloudiness, accompanied by loss of potency, has been observed to become developed, by keeping them at about 50° C. for ten days. The instability of insulin in the presence of alkali is of interest, and has been systematically investigated by Witzemann and Livshis. By standing at room temperature for six days in the

presence of 0·5N . NH_4OH, insulin all but loses its potency, which, however, gradually returns if the solution be made acid again, which has led these investigators to suggest that some tautomeric change occurs in the insulin molecule. Alkaline phosphates and carbonates do not appreciably affect the strength of insulin, even on prolonged standing at room temperature.

The proteolytic enzymes rapidly inactivate insulin (trypsin, pepsin, papain, and erepsin), and it is generally believed that this is because it is destroyed. Epstein and Rosenthal have, however, made the statement that this is really not the case, but that the insulin merely becomes inactivated, and that its potency can be restored by raising the acidity of the solution. They state that this inactivation occurs promptly when trypsin is added to insulin in faintly alkaline reaction *in vitro*, and that it also occurs *in vivo* when trypsin is injected along with insulin. They draw far-reaching conclusions regarding the rôle that such a combination occurring in the body must have in the ætiology of diabetes. But other investigators have failed to corroborate their results, and they seem, inherently, to be highly improbable.

An attempt to reduplicate them in my laboratory (by Macela) seemed, at first, to be successful, but not so when regard was taken of the behaviour of insulin towards weak alkali. As a matter of fact, insulin in the presence of weak alkali and trypsin seems to be rapidly and permanently destroyed, which is the basic fact upon which depended the conclusive demonstration (by Banting and Best) of its presence in pancreatic extracts.

It is possible that insulin may exist in the cells of the islets of Langerhans as some inert compound prior to its secretion into the blood, or at least as some precursor which is activated during the process of extraction, but it is highly improbable, as has been suggested by Epstein and Rosenthal, that trypsin plays any rôle in this connection.

Much attention has been paid as to whether or not insulin gives the colour reactions for proteins. When insulin from the ox or pig pancreas is used, the biuret test is invariably obtainable, although I have failed to observe it in insulin prepared from the pancreas of the skate (Raja), and Murlin and his collaborators have failed to obtain it in ox insulin prepared by them (p. 72). This may merely mean that the biuret test is not sensitive enough. Doisy, Somogyi, and Shaffer still obtained this reaction, as well

as that of Millon, and Dudley is emphatic that it always occurs. With regard to the other colour reactions, there is much division of opinion, and an excellent summary of the findings will be found in the article by Grevenstuk and Laqueur. Until insulin can be prepared in greater purity, however, it is useless to place any weight on these reactions. The suggestion has been made that insulin may be related to guanidin (Sjollema and Seekles (cf. Grevenstuk and Laqueur)). J. J. Abel and his co-workers have recently succeeded in obtaining insulin in crystalline form.

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CHAPTER VII.

THE CLINICAL HISTORY OF DEPANCREATISED DOGS TREATED WITH INSULIN.

HAVING reviewed the experimental evidence upon which depends the theory, that the islets of Langerhans control the metabolism of sugar in the animal body through the internal secretion of insulin, we will now proceed to examine more closely the effects which follow when insulin is given to depancreatised animals. The object in view in such investigation is that some light may be thrown on the problem of the physiological rôle which insulin plays in the control of metabolism. We shall first of all study the clinical history of completely depancreatised animals kept alive by daily administration of the hormone, and then consider each in turn, certain of the diabetic symptoms in relationship, not only to the effects of insulin on them, but also to the broader question of their significance in connection with the nature of the diabetic state.

The investigations have been almost exclusively confined to dogs, since, for various reasons, other animals are not suitable. Although pancreatectomy can readily be performed on cats, it is unusual for the animal to keep in good condition after it, and very difficult to collect samples of blood for determination of the sugar concentration, or to conduct accurate observations on the metabolism. In rabbits the operation itself is impossible, because of the spread-out condition of the pancreas. This restriction of the observations to dogs has had the effect of limiting them in number with regard to those which demand the sacrifice of the animal, such, for example, as determination of the glycogen and fat content of the tissues.

Although diabetes in man is probably always associated with a diminished internal secretion of insulin from the pancreas, only relatively few cases are so acute as to be comparable with the condition which is set up in dogs by complete pancreatectomy.

A type of experimental diabetes very like the clinical can be established by partial removal of the pancreas (p. 51), and much of importance has been learned regarding the human disease by experimental studies on this condition. Both in it and the clinical forms, however, there are essential differences from diabetes due to complete pancreatectomy, for in both of those types there still remains enough acinar tissue to secrete some of the digestive juices of the gland, and a few healthy islets remain to secrete insulin into the blood. There may even be some other internal secretory function, besides the production of insulin, subserved by the pancreas.

It will be recalled that Banting and Best succeeded, by almost daily injections of the various pancreatic extracts prepared by them, in keeping a depancreatised dog alive for a period of seventy days, when the animal was killed by chloroform. No pancreas could be seen post-mortem, but nodules of pancreatic tissue were found by W. L. Robinson in the walls of the duodenum.

After insulin became available, observations of a similar nature were immediately undertaken, in collaboration with Allan, Bowie, Markowitz, and others, with the object of throwing light on several questions, among which may be mentioned the following: (1) Can a depancreatised animal be maintained in a perfectly healthy condition without any other pancreatic function available to it than that conferred by the injection of insulin. If any function should continue to be imperfectly performed under these conditions, it might be possible to determine whether this was owing to the absence of some other endocrine function of the pancreas than the secretion of insulin, or merely to the serious upset of the digestive process, entailed by removal of its external secretion; (2) Can some of the lost power to metabolise carbohydrate be reacquired after a time. In other words, can some other organ, or tissue, functionate vicariously for the missing insular cells, and thereby come to produce insulin? (3) How long does it take for an adequate supply of insulin in the body to become exhausted? (4) What is the relationship between the dose of insulin given the depancreatised animal and the amount of glucose which it is thereby enabled to metabolise?

In the first animals which were treated with insulin, the diet consisted exclusively of moderately lean meat, fat being withheld

because when given most of it passed, undigested, with the fæces. Enough insulin was also given, twice daily, to maintain the urine sugar free. These animals did not thrive: they lost in weight however much meat was given, the skin became unhealthy, and the observations had to be terminated after a month or two. Obviously, enough food was not being assimilated. This led us to add cane sugar to the diet, along with a sufficient increase in the dose of insulin so that a trace of glucose was excreted in the urine. This plan was adopted, in preference to one in which just enough insulin was given to prevent glycosuria, so as to avoid the danger of an overdose, the symptoms of which might develop during the night, thereby increasing the risk of losing the animal. The immediate outcome of the addition of sugar was that the animals put on weight and became apparently normal in every regard. By altering the proportion of sugar in the diet the body weight could usually be caused to increase or decrease at will. Several animals thus treated were used by F. N. Allan for the purpose of determining the glucose equivalence of insulin, that is, the number of grammes of glucose which each unit of insulin could cause to be metabolised. An account of these observations will be found elsewhere (p. 96). But this modification did not succeed in keeping the animals alive for more than a few months (two to seven), death being due to a complete upset of the hepatic function. The clinical picture is well illustrated in the following protocol of the second animal in which the symptoms were observed.

Female, weighing to start 9·2 kg., was depancreatised on 13th February, 1923, after which it was given insulin twice daily, and fed at first with various types of diet, and later with lean meat and cane-sugar. The amount of meat was 500 gms. daily, along with 100 to 150 gms. cane-sugar, although towards the end of the observation the meat was increased to 900 gms. Glycosuria was constantly present, the degree of which varied according to the dose of insulin. The animal remained in good condition until 12th April (two months after the operation), when, in contrast to its previous behaviour, it appeared depressed and apathetic, and took little food. The rectal temperature was 40° C. Next day the dog refused food, and jaundice was noticed in the sclera and the skin inside the ears. Bile-pigment was found to be present in the urine, the volume of the latter becoming less and less, so that the concentration of bile rose very greatly. On 14th April the dog was very weak and apathetic. During the day it became extremely feeble and the respirations rapid and somewhat distressed; it died

during the following night. In the cage were found dark, semi-fluid fæces containing a small amount of red blood.

Post-mortem.—The liver was large and very friable, and was of a pale yellowish nutmeg appearance. No obstruction could be detected in the biliary tract. The gall-bladder contained about 5-10 c.c. of bile. The kidneys and spleen appeared to be normal.

In three other animals similarly treated, the clinical history and the gross pathological findings were very similar to those just described, and in all of them microscopic examination of the liver revealed an extreme degree of fatty deposition. The fat was deposited as very large globules in the cells of the periphery of the lobules, while those at the centre showed evidences of cellular degeneration with less of the fatty changes. In the liver of one of the animals, as much as 39·5 per cent. of fat was found to be present. Not only is this decidedly more than the amount found by us in the livers of depancreatised dogs not treated with insulin, but its distribution in the lobule was different, for in the non-insulin cases the fat deposits are decidedly richer in the cells towards the centre of the lobules, and these cells are swollen and compress the sinusoids. No significant morbid changes could be detected in any other organ or tissue of these animals, except that in one of them there was evidence of nephrosis.

There can be no doubt that a complete breakdown of the hepatic function was the cause of death in all these cases, and the question arises, whether some toxic substance, either introduced with the insulin from without or arising in the body, may have been responsible. There is plenty of evidence to indicate that the insulin itself was not the cause, for not only are there now many patients who have received it daily for four years, but, as we shall see later, depancreatised dogs have been kept under it for over two years without showing any similar symptoms. Evidently some toxic condition developing within the body was responsible for their occurrence, and, moreover, this must have been of a local nature, since the liver alone showed evidence of its effects, the nephrosis observed in one of the animals being almost certainly only secondary to the hepatic change. In some way or another, the continued absence of the pancreas leads to a pathological condition in the liver cells, and as a result, fat accumulates in them. This does not occur in diabetes in man, even in the most severe cases, because some of

the pancreas still remains intact. It is the entire absence of the gland that is responsible, and there are two possible explanations for the effects : either, that the digestive process in the intestine becomes of an abnormal type, particularly with regard to the digestion of proteins, so that amines, or similar toxic substances are produced, which are then carried by the portal blood to the liver ; or, that the pancreas, quite apart from its function in producing insulin, also secretes some other hormone which is necessary for the physiological integrity of the liver cell. In favour of the former of these possibilities may be mentioned the fact, that about 50 per cent. of the ingested protein, besides practically all of the fat, reappeared in the fæces, which contained much of the meat in an undigested state, as revealed by microscopic examination. Neither could starchy foodstuffs be properly digested (dog biscuit), as evidenced by their causing violent diarrhœa.

In connection with the possibility that the pancreas secretes some other hormone besides insulin, it is interesting to suppose that this may have to do with the chemical changes which fatty acid undergoes in the liver cells, prior to its utilisation in the tissues. Indeed, one may imagine that this other hypothetical hormone is lipase, or some closely related esterase. Lombroso, several years ago (1910), suggested that the pancreas has some influence on the absorptive function of the intestine, perhaps through an internal secretion, for he found that whereas depancreatised animals still retaining some pancreatic tissue, as a graft, could assimilate 80 per cent. of ingested fat, they failed to assimilate any when the graft was removed. McClure, Vincent, and Pratt (1916), however, have failed to corroborate this finding.

These considerations of the cause for the fatty changes in the liver suggested the addition to the diet of raw pancreas in sufficient quantity so that some of it might pass through the stomach before its digestive enzymes had time to be destroyed. This was done for the first time during November, 1923, and immediately it was noted that the food was better assimilated, so that the addition became the rule for all the depancreatised animals under observation. Two of these animals have been of especial interest. One of them was depancreatised on 22 November, 1923, and the other on 14th February, 1924, and they are both alive and in apparently normal condition at the present

date (September, 1925); therefore after twenty-two months and nineteen months respectively. Both animals were depancreatised by Dr. F. N. Allan, and were used by him, partly to determine the glucose equivalence of insulin, and, along with Dr. Sokhey, to study the behaviour of the urinary phosphates (p. 330). The body weights could be made to alter by varying the amount of cane sugar in the diet, and both animals have at some periods been unusually fat, and at others, unusually thin. Absorption of protein, and of fat, has been conspicuously more satisfactory than in animals not receiving pancreas. One of the animals gave birth to six pups without there being any change in the carbohydrate balance during the pregnancy, although violent symptoms, due to hypoglycæmia (p. 276), supervened on the day after parturition, because of the withdrawal of sugar in the milk. The abbreviated history of this animal is as follows :—

14 February, 1924.—Depancreatised by Dr. F. N. Allan and put on diet of 400 gms. meat, 50 gms. sucrose, and given 16 units insulin (twice daily).

27 February, 1924.—Insulin discontinued and dog given 200 gms. meat twice daily. As a result it showed gradually increasing weakness, became very thirsty, and passed large volumes of urine containing much sugar and acetone bodies.

4 March, 1924.—Insulin given, and dog used for determining relative absorption of fat in absence of pancreatic lipase.

28 May-30 July, 1924.—Used for insulin assay work.

7 June, 1924.—Fresh pancreas added to the diet of meat and sugar.

8 June, 1924.—Weight 16 lb., same as before pancreatectomy.

22 October, 1924.—Showing usual signs of œstrus.

24 October, 1924.—Put on a constant diet of 200 gms. meat, 50 gms. sucrose, 50 gms. pancreas, and 16 units of insulin twice daily, at 10 a.m. and 5 p.m. This diet, as well as insulin dosage, was maintained throughout the remainder of the observations.

26-28 October, 1924.—Put in room with male dogs.

12 November, 1924.—Weight, 22 lb. 13 oz.

1 December, 1924.—Abdomen and mammary glands enlarging, general condition excellent.

4 December, 1924.—Weight, 23 lb. 12 oz.

17-22 December, 1924.—Some symptoms of mild respiratory infection.

31 December, 1924.—Post-absorptive blood sugar, 0·429 per cent., general condition excellent.

1 January, 1925.—9 a.m., four pups born, one dead ; 4 p.m., fifth pup born, dead ; 4.25 p.m., sixth pup born, dead.

2 January, 1925.—Three pups and mother in good condition. About mid-day became excited and soon lost interest in pups. Breathing

gradually became hyperpnœic and salivation occurred. It was clear that the animal was exhibiting symptoms of hypoglycæmia, and the blood was found to contain only 0·067 per cent. glucose.

Because it illustrates well the normal state of other similarly treated depancreatised animals, and also because of the special interest attaching to this one, the metabolic balances (as determined by W. W. Simpson) are given in Tables III. and IV.

In view of the development of symptoms of hypoglycæmia on 2nd January, which was the day following the birth of the pups, the blood sugar was determined, for the next few days, at the same time each day. The animal was kept on the standard diet, and the insulin dosage was not changed during these days, with the exception that the 5 o'clock meal on 2nd January was half the usual one along with only five units of insulin. She continued to suckle the two pups.

Date.	Time of taking Blood.	Blood Sugar.
January 2	3·40 P.M.	0·067
,, 3	3·45 ,,	0·083
,, 5	3·30 ,,	0·135
,, 6	3·40 ,,	0·250
,, 8	3·40 ,,	0·105

Besides taking blood in the afternoon, post-absorptive bloods were taken before the morning meal :—

Date.	Post-absorptive Blood Sugar.
January 5	0·433
,, 7	0·422
,, 10	0·338
,, 16	0·220

From 1st January to 16th February the mother was suckling the two pups, and as these were in the cage with her, it was not possible to make observations on her nitrogen metabolism.

The urinary sugar values for twenty-four hour periods were as follows :—

Date.	Urinary Sugar.
January 4	51·10 gms.
,, 5	52·02 ,,
,, 6	51·62 ,,
,, 16	3·88 ,,
,, 17	1·31 ,,
,, 18	2·39 ,,
,, 19	2·22 ,,
,, 28	0·54 ,,
,, 29	3·43 ,,
,, 30	4·05 ,,

February 1, pups weaned.

TABLE III.

Date.	Weight.	Urine. N.	Fæcal.* N.	Total. N.	Glucose † from Protein.	Glucose ‡ from CHO.	Total Glucose Intake.	Glucose Excreted.	Glucose Utilised.
	lb.	gm.	gm.		gm.	gm.	gm.	gm.	gm.
Oct. 25	—	14·06	—	—	88·07	105·3	193·37	29·9	163·47
,, 26	—	12·46	—	—	77·88	105·3	183·18	34·0	149·18
Nov. 24	23¼	13·61	1·121	14·73	85·06	105·3	190·36	20·80	169·56
,, 25	23¼	13·81	1·121	14·93	86·31	105·3	191·61	23·40	168·21
,, 26	23¼	13·32	1·121	14·44	77·25	105·3	182·55	41·58	140·97
Dec. 9	25	13·74	1·605	15·34	83·25	105·3	188·55	29·90	158·65
,, 10	25½	12·72	1·605	14·33	79·50	105·3	184·80	27·50	157·30
,, 11	25¼	12·55	1·605	14·16	78·44	105·3	183·74	48·45	135·29
,, 16	26¼	12·69	0·7502	13·44	79·32	105·3	184·62	41·29	143·33
,, 17	26¼	11·18	0·7502	11·93	68·87	105·3	174·17	49·60	124·57
,, 18	26¼	10·15	0·7502	10·90	63·44	105·3	168·74	37·85	130·89
,, 22	27¼	11·80	1·10	12·90	73·75	105·3	179·05	30·45	148·60
,, 23	27½	11·60	1·10	12·70	72·40	105·3	177·70	40·92	136·78
,, 24	28	11·12	1·10	12·22	69·50	105·3	174·80	41·36	133·44
,, 25	28	11·32	1·10	12·42	70·65	105·3	175·95	25·00	150·95

* Fæces were collected over a number of days and a daily average nitrogen content estimated. Charcoal feeding was employed in determining the starting and end points of collection.

† Urinary nitrogen × 6·25.

‡ Sucrose of diet × 1·053.

It was now possible to resume the nitrogen determinations. Sugar and nitrogen balances for March are given in Table IV.:—

TABLE IV.

Date.	Weight.	Urine. N.	Fæcal. N.	Total. N.	Glucose from Protein.	Glucose from CHO.	Total Glucose Intake.	Glucose Excreted.	Glucose Utilised.
	lb.	gm.	gm.		gm.	gm.	gm.	gm.	gm.
Mar. 3	—	11·8	—	—	74·19	105·3	179·49	3·34	176·15
,, 4	—	12·75	—	—	79·69	105·3	184·99	3·82	181·17
,, 5	—	12·65	—	—	79·06	105·3	184·36	6·00	178·36
,, 6	—	12·23	—	—	76·44	105·3	181·74	40·40	141·34
,, 7	—	11·48	—	—	71·75	105·3	177·05	25·97	151·08
,, 22*	22	10·14	—	—	63·38	105·3	168·68	27·36	141·32
,, 23	22	14·17	—	—	88·56	105·3	193·86	23·75	170·11
,, 24	22	12·87	—	—	80·44	105·3	185·74	26·15	158·59

* Dog back to pre-pregnant weight and health.

The metabolic balances are displayed in the chart of Fig. 11, from which it can be seen that as the weight increased the urinary excretion of nitrogen diminished (that of the fæces remaining constant) due, no doubt, to nitrogen retention by the growing fœtuses.

Perhaps the greatest interest of these results is that the sugar balance remained undisturbed throughout the entire pregnancy. This would seem to stand at variance with the findings of Carlson, Drennan, and others, that complete pancreatectomy in pregnant bitches near full term is not followed by glycosuria (Carlson, Orr, and Jones, 1914) or hyperglycæmia (Carlson and Ginsburg, 1914) as long as the fœtuses are alive and the placental connections intact (Carlson and Drennan, 1911), and to contradict the conclusion which they drew, that the internal secretion (of insulin) from the growing pancreas of the fœtuses could protect the mother against the consequences of pancreatectomy. The results just recorded certainly do not lend support to these views, although the possibility still remains, in the light of the work of F. N. Allan (1924) with regard to glucose equivalents (p. 96), that the amount of insulin which could be secreted by the fœtuses would be too small to make any measurable impression on the glucose balance of the mother, on account of the large amount of insulin injected from without.

Another fact of great interest which these investigations

bring to light is that serious symptoms of hypoglycæmia may supervene after parturition when the mammary glands start to secrete milk, the cause being, undoubtedly, the withdrawal of glucose from the maternal organism, to form the lactose of the milk.

Shortly after this observation was made by us, similar ones were recorded by Widmark and Carlens (1925) in the case of calving cows, and shown by them to be the cause of so-called "Milk Fever." The blood sugar of milking cows, while on

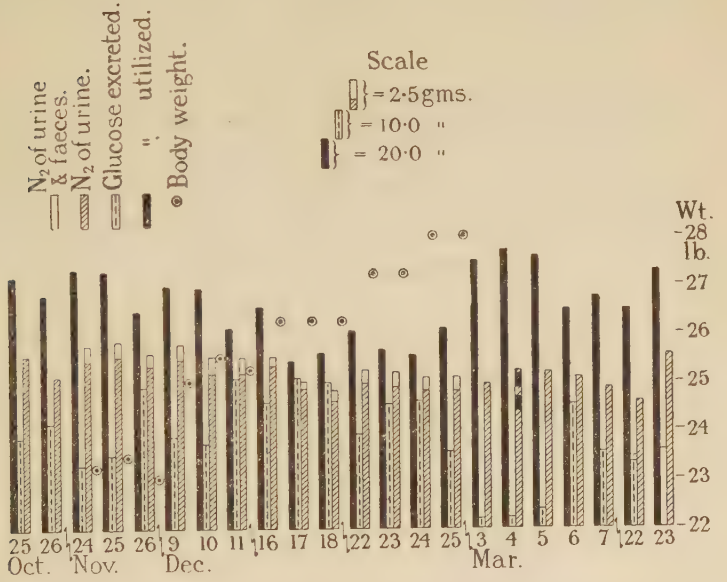

FIG. 11.—Chart showing metabolic balances during the course of pregnancy in a depancreatised dog treated with insulin. (W. W. Simpson.)

ordinary green food (beet leaves), is decidedly lower than that of other domestic animals (average, 0·085 per cent.), the lowest values being obtained in the case of cows giving the most milk. The symptoms of milk fever, which are common just after calving, are very similar to those which can be induced by injecting insulin (500 units) into a normal animal, but they do not supervene until the blood sugar has fallen to well below 0·040 per cent. The well-known treatment for milk fever—injecting air into the udder—is believed to act by stopping the milk secretion, so that the blood sugar rises again, and Widmark and Carlens

have shown that a similar operation performed on normal cows causes a marked degree of hyperglycæmia to become developed, accompanied usually by the appearance of glucose, and also of lactose, in the urine. It would be interesting to know if injection of air subcutaneously elsewhere than into the udder would also affect the blood sugar, because of reflex stimulation of glycogenolysis. It has also been found that the injection of glucose solutions into affected animals removes the symptoms of milk fever, but the quantities injected must be considerable, because of the very large drain of sugar through the milk (about 60 gms. per hour).

There is nothing else with regard to the condition of these two insulin-treated depancreatised dogs to which reference need be made here. Both are now on the retired list, and are being carefully treated so as to ascertain for how long it may be possible to keep them in perfect health, occasional determinations of their carbohydrate balances being made, to find out whether any of the lost power to metabolise carbohydrate may be restored. So far both animals have been found to excrete precisely the same quantities of glucose as they excreted while under the same treatment during the first few months following removal of the pancreas. This is well illustrated in the protocols of dog 23 (Tables I. and II.), the daily average carbohydrate balance and the body weight being the same at the end of March, 1925, as at the beginning of October, 1924. It may be remarked that this constancy in the carbohydrate balances bears testimony to the dependability of the rabbit assays of the various batches of insulin which were used from time to time (see also p. 342). The apparently normal condition of these animals, despite the high level to which the blood sugar rises during certain periods of each day (post-absorptive), would seem to contradict the view of clinical observers that hyperglycæmia *per se* can be the cause of degenerative changes in the arteries, the kidneys, the eyes, and other tissues. No indications of such changes have been observed, nor do the animals appear to be susceptible to those catarrhal infections which are often observed in dogs kept in confinement.

On several occasions, in both these animals, insulin and food have been withdrawn for a day or so, and invariably with the

same results: the very rapid return of severe symptoms of diabetes. By the second day following the withdrawal, especially in fat animals, not only does glycosuria become extreme, but ketonuria very marked, accompanied by symptoms of great depression, drowsiness, vomiting, and rapid breathing. This rapid return of symptoms when insulin is withdrawn indicates that no other organ or tissue can replace the pancreas as the source of this hormone in the animal body.

Pancreatic nodules have been found by Bowie in the duodenum of both normal and depancreatised dogs, but no islets have been observed in them.

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CHAPTER VIII.

THE EFFECTS OF INSULIN ON THE METABOLISM OF DEPANCREATISED DOGS.

In the present chapter we will consider some of the chemical changes occurring in the blood and urine, and in the tissues of depancreatised animals treated with insulin, as described in the preceding chapter. Because of the marked changes which rapidly result in the sugar, the ketone bodies and the phosphates of the blood when insulin is given, or is withheld after having been given for some time, much can be learned of the relationship of these substances to one another in metabolism. By the latter method a much more favourable condition is provided for the investigation of these problems than offers when freshly depancreatised animals are used, for not only is the post-operative shock entirely avoided, but animals can be brought into any desired condition of "fatness" before discontinuing the insulin, and the relationship of body fat to the gravity of the diabetic symptoms accurately observed.

The Effects of Withdrawal of Insulin on the Blood.—As has already been pointed out in the previous chapter, withdrawal of insulin and food soon brings the animals into a very low state, with symptoms not unlike those of diabetic coma in man. In Table V. are given the amounts of sugar, inorganic phosphate, β-oxybutyric and acetoacetic acids and fat which were found in the blood by Chaikoff and Markowitz. The animals are designated as fat or thin, observations being made on three separate occasions on each of the first two of them, A and B. It is evident that the blood sugar was on an average decidedly higher in the fat, as compared with the thin animals, a similar difference being also evident with regard to acetoacetic acid and, less distinctly so, with regard to oxybutyric acid. In the fat animals also the acetoacetic acid was present in greater amount than β-oxybutyric,

whereas in thin ones this relationship is reversed. With regard to fat and phosphates, the results do not permit of definite conclusions.

TABLE V.

FAT AND OTHER DOGS.

Dog.	Nutritive Condition.	Duration of Deprivation of Insulin.	Blood Sugar.	Inorganic Phosphorus of Blood.	β-hydroxy Butyric Acid of Blood.	Acetoacetic Acid of Blood.	Fat of Blood.
		Days.	mg. per 100 c.c.	mg. per c.c.	mg. per c.c.	mg. per c.c.	mg. per c.c.
Dog A	Thin	3	0·380	5·0	0·27	0·044	18·6
,,	,,	6½	0·359	—	0·070	0·064	—
,,	,,	6	0·423	4·2	0·21	0·12	—
Dog B	Fat	3	0·438	6·4	0·23	0·32	10·3
,,	,,	5	0·600	4·98	0·41	0·86	9·5
,,	,,	3	0·700	5·89	—	0·53	11·7
Dog G	Very fat	2½	1·38	8·3	0·70	1·5	15·0
Dog M	Thin	4	0·468	—	0·48	0·45	5·5
Dog T	Medium	3	—	—	0·12	0·063	—

These observations were undertaken at the intervals following withdrawal of insulin which are indicated in the table, these being, as far as we could judge, as long as it was safe to withhold the insulin. Indeed, certain of the animals, notably Dog G and Dog B (last observation), died during the night following the observations. Before discussing the significance of these results, we will study the effects produced by again giving insulin.

The Effects of Re-administration of Insulin on the Blood.—Typical results of such an observation will be seen in Fig. 12. The animal (A of Table V.) was depancreatised about six weeks before the experiments, and three days prior to the observation insulin was discontinued. On the morning of 15th October, the sugar, phosphates, oxybutyric acid, and fat were all considerably above the level for normal animals. Twenty units insulin were given at 10.20 A.M., and during the next three observations, which were made after intervals of approximately 40, 70, and 100 minutes, the sugar, the phosphates and the oxybutyric acid decreased at almost exactly the same rates, this being independent of dilution of the blood, which, however, occurred later to a certain extent. After this time the fall in phosphoric acid and β-oxybutyric acid became very much less, although that of sugar continued at almost its original rate. The two former also began to recover earlier than the latter. There was no

definite decrease in fat, the oscillations in this being, we think, dependent on uncertainties in its determination. The animal on which these observations were made was considered lean for its type, its weight at the end of the experiment being 7·3 kg.

In another animal (B. of Table V.) that was decidedly fat, and in which complete pancreatectomy had been performed, by removal of a pancreatic graft three weeks before the observation,

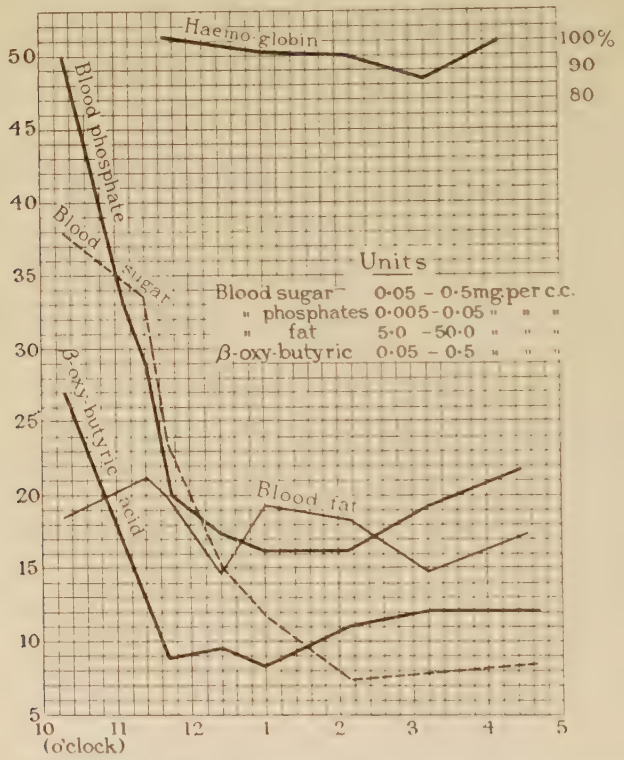

FIG. 12.—Chart showing effect of insulin on blood constituents in depancreatised dog. (From Chaikoff, Macleod, Markowitz, and Simpson, "Amer. Jour. Physiol." 1925.

twenty-five units of insulin reduced the sugar, phosphates, and acetoacetic acid at approximately the same rates for the first 100 minutes following the injection. After this the phosphate quickly recovered, although the sugar continued steadily to fall, until it reached 0·29 per cent. The acetoacetic acid also steadily decreased until in about five hours it was only 7 mg. Blood fat in this animal, as in the previous one, did not perceptibly change

during the time of the observation, and the hæmoglobin increased to 108 per cent. over its initial value. In another observation on the same animal some weeks later, similar results were obtained, except that the blood sugar apparently began to recover at the same time as the phosphates.

The parallelism between the fasting blood sugar and the amount of body fat points to the possibility of a close relationship between them; it supports the view that sugar is derived from fat, at least in the diabetic organism, and the relatively greater concentration of the ketone bodies, especially acetoacetic acid, further suggests that these occur as intermediate products in the change. Other evidence for this view has recently been strongly put forward by Geelmuyden, and we know of no fact which irrefutably contradicts it. Nevertheless, in considering these observations in its support, the possibility must not be lost sight of that the excess of sugar in the fat animals may be dependent on their possessing relatively richer glycogen stores in the liver than the thin ones. This possibility awaits further investigation, but as opposed to it, may be mentioned that we have found that the respiratory quotient (R.Q.) ceases to become raised by the ingestion of sugar at the same interval after discontinuing insulin in the fat as in the thin animals. If this can be taken as an indicator of the exhaustion of the glycogen stores, as is commonly believed to be the case (see p. 51), the results of these observations furnish very strong evidence for the derivation of sugar from fat.

The behaviour of phosphoric acid following insulin is in no way different from that in normal blood (p. 332), but it is interesting to note that the percentage prior to administration of insulin is much higher, which corresponds with the higher amount in the urine. It will be convenient, however, to defer a further discussion of the behaviour of the phosphates in diabetic animals until we have studied the changes which they undergo in normal animals after the injection of insulin (p. 51).

The Behaviour of the Alkali Reserve of the Blood.—Since the ketone bodies of the blood diminish so promptly following insulin, it is to be expected, in cases where the ketosis is so marked that there is a decided lowering of the alkaline reserve, that this will rise as the ketone bodies fall. This phase of the problem has not been systematically investigated in dogs, because of the

much greater convenience with which it can be done in human patients, and an account of this work will be found elsewhere (Campbell and Macleod, 1924). Most clinical observers believe that the rate at which the ketone bodies disappear, following the injection of insulin in diabetes, is increased when sugar is given at the same time, and it is a common custom to use 1 gm. glucose for each unit of insulin. Under these conditions, as the ketone bodies disappear, the alkaline reserve (CO_2 combining

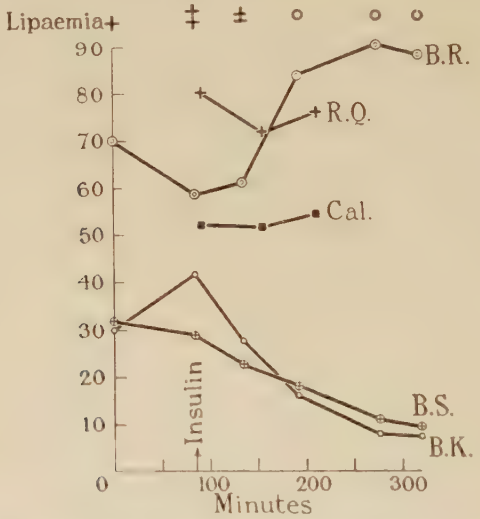

FIG. 13.—Chart showing effect of insulin on alkaline reserve, ketone bodies, etc., in blood of diabetic patient (see text). B.R. = Bicarbonate reserve percentage (normal, 100 per cent.). R.Q. = Respiratory quotient. Cal. = Calories per hour calculated from respiratory exchange. B.S. = Blood sugar percentage. B.K. = Blood ketone bodies in milligrams per cent. The respiratory quotient and the blood sugar percentage have been multiplied by one hundred in order to show them by means of the same scale of ordinates. Abscissæ = time in minutes from commencement of observation. Patient fasting; weight, 32·3 kilos. Insulin, 30 units at point shown. (From H. W. Davies, C. G. Lambie, *et al.*, "Brit. Med. Jour.," 1923, 1847.)

power) of the blood, as measured by the Van Slyke method, rises very rapidly, as is shown in the curve of Fig. 13 (taken from a paper by Davies, Lambie, Lyon, Meakins, and Robson, 1923), in which B.R. represents bicarbonate reserve; B.K., the ketone bodies of the blood; B.S., the blood sugar; Cal., the calorie output; and R.Q., the respiratory quotient. The degree of lipæmia is also indicated by the signs of "+" or "O" at the top of the chart. The case was one of severe diabetes in a woman

with a carbohydrate tolerance, at the time of observation, of only 10 gms. a day. The amount of insulin given was thirty units, and it will be observed that this caused the B.R. to rise from 59 per cent. to 91 per cent. of the normal in less than three hours.

The Effect of Insulin on Ketonuria.—With regard to the effect of insulin on the excretion of ketone bodies in the urine, the most satisfactory observation can be made on diabetic patients, in whom this excretion is usually much greater than is the case in depancreatised dogs. The effect of insulin on this excretion is shown in the accompanying curve (Fig. 14), from a case observed by Banting, Best, Collip, Campbell, and Fletcher (1922).

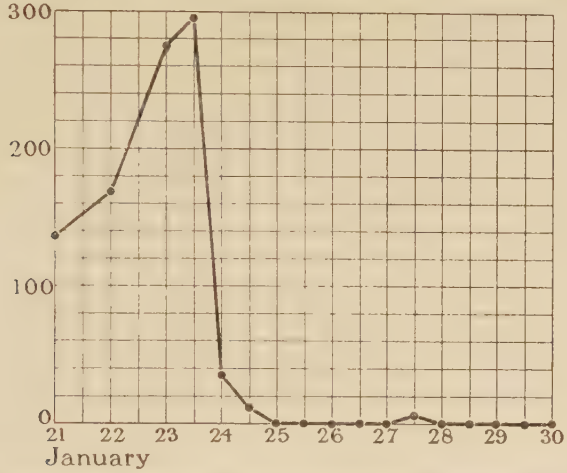

FIG. 14.—Showing the cessation of ketonuria following administration of extract. (From Banting, Best, Collip, Campbell, and Fletcher, "Can. Med. Assn. Jour.," 1922, xii, 141.)

In depancreatised dogs under certain conditions, however, the acetone excretion may be considerable, and insulin also promptly removes it, as is shown in the table on page 96, drawn up from results obtained by J. B. Collip from one of the earlier observations in which insulin was used on animals, prior to its use in the treatment of diabetes in man.

It will be seen that in the urine collected on the day after insulin the acetone bodies entirely disappeared, although there was still a considerable excretion of sugar. They remained away for two days more, and then reappeared on the fourth day. If we assume, as is commonly done, that ketonuria only

TABLE VI.

EXCRETION OF SUGAR AND KETONE BODIES IN DEPANCREATISED DOGS.

Date.	Insulin Given.	Total Urine.	Total Dextrose Excretion.	Total Acetone Bodies.	Remarks.
		c.c.	gm.	mgm.	
Jan. 6	No	1000	29·7	100	—
,, 7 A.M.	,,	375	28·4	187	Blood sugar 0·351 per cent.
,, 7 P.M.	Yes	—	—	—	,, 0·085 ,,
,, 8 ,,	No	425	4·25	None	—
,, 9 ,,	,,	325	9·95	None	—
,, 10 ,,	,,	370	9·6	None	—
,, 11 ,,	,,	275	25·2	34	—
,, 12 ,,	,,	325	25·4	55	—
,, 13 A.M.	,,	750	18·0	114	—
,, 13 P.M.	Yes	—	—	—	—
,, 14 ,,	,,	600	8·0	None	—

occurs when the glycogen of the liver has disappeared, this result can be explained as dependent on the fact that glycogen became formed in the liver as the consequence of the injection of insulin, and remained available for several days after insulin was discontinued.

The Amount of Glucose which Insulin can cause to be Metabolised. The Glucose Equivalent of Insulin.—Since there is no insulin being secreted in the body of a completely depancreatised animal (p. 89), the amount of glucose which each unit of insulin can cause to be metabolised may be determined by deducting the total excretion of glucose in a fixed period of time from the amount of glucose available in the food, and then dividing by the number of units of insulin meanwhile administered. The glucose of the food includes the carbohydrate actually given, and the amount which may come from protein, this being reckoned on the basis that 58·6 per cent. (60 per cent.) may be so derived (see p. 109). This factor is based on the D : N ratio of 3·65, obtained by Lusk and others in the case of starved or meat-fed phlorhizined dogs, and may not apply in the case of animals receiving food which contains carbohydrate (p. 107). The proportion of glucose derived from protein may be much less than 3·65, especially since, under insulin, carbohydrates must have a protein-sparing action. It is recognised, therefore, that the method for calculating the total glucose intake is an arbitrary one, but notwithstanding this, the results obtained by Frank N.

Allan (1924), are of great interest, and bring to light several very interesting facts regarding the glucose equivalents of insulin. These facts are as follows: (1) The equivalent is remarkably

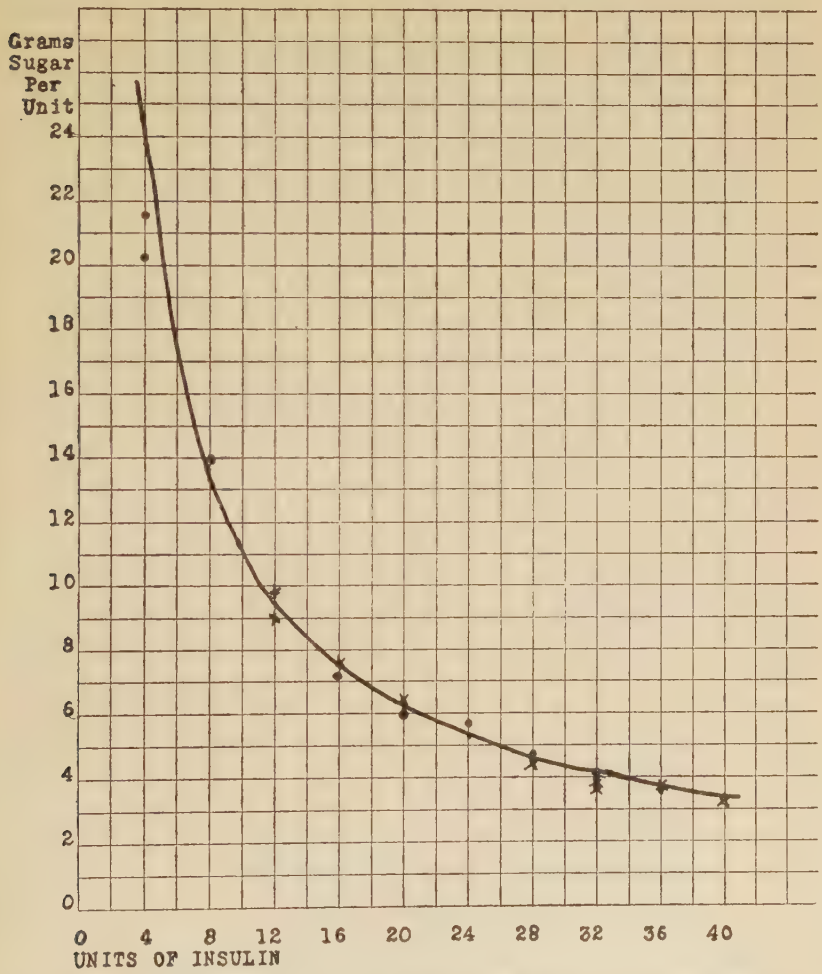

FIG. 15.—Curve showing glucose equivalents: for Dog I. on 100 gms. sugar (represented by dots) and for Dog J. on 100 gms. (represented by crosses). (From Frank N. Allan, "Amer. Jour. Physiol.," 1924, lxvii, 275.)

constant, both for the same and for different animals, when the carbohydrate balance is at the same level; (2) it is much higher with small doses of insulin than with large ones, the carbohydrate intake being the same. That is to say, when little carbohydrate

is being metabolised, as is the case when no insulin is given, the administration of one unit will have a much greater effect in causing glucose to be metabolised than when the one unit is given to an animal in which, by previous insulin administration, considerable combustion of glucose is already proceeding. As illustrating these facts, the following figures and the curve of Fig. 15 are of interest :—

DOG I.

FED DAILY ON 600 GRAMMES LEAN MEAT AND 100 GRAMMES SUCROSE.[1]

Insulin Given Daily (clinical units).	Glucose Equivalents (on different days).
20	5·8, 5·8
24	4·1, 4·0
32	3·5, 3·5, 3·6, 3·3, 3·7 (4·1)
36	3·0, 3·1, 3·1
38	3·4
40	3·0, 3·3, 3·1, 3·0

There are probably several reasons for the progressive decline of the value of the glucose equivalent as the dosage of insulin is increased. One of these is undoubtedly that when a relative excess of insulin is given some is either lost in the urine or is destroyed in the body. This, however, cannot be the only reason, because the same differences are observed when the glucose equivalents are determined for different periods in which the amount of insulin is kept constant, but the amount of sugar given daily is increased. Thus :—

DOG I.

FED ON 600 GRAMMES LEAN MEAT, BUT WITH VARYING AMOUNTS OF SUCROSE.

A. Insulin Given Daily, 20 Units.

Sucrose Given Daily.	Glucose Equivalents.
50 grammes	3·5, 3·9
100 ,,	5·8, 5·8
150 ,,	8·4, 8·1, 6·9, 6·9

B. Insulin Given Daily, 32 Units.

Sucrose Given Daily.	Glucose Equivalents.
50 grammes	2·4, 2·6
100 ,,	3·7, 4·1
125 ,,	4·9, 4·8
150 ,,	4·5, 4·9

[1] To illustrate the method of calculation it may be well to give an example. Let us suppose that with 20 units of insulin and a total glucose intake of 145 gms. the output of glucose by the urine is 35 gms. (utilisation, 110 gms.), and that with 30 units, but the same total glucose intake, the output falls to 5 gms. (utilisation, 140 gms.), then the equivalent, when 20 units are given, is 5·5; and it becomes 4·7 with 30 units.

This indicates that the amount of glucose which each unit of insulin can cause to be utilised will depend on the relative number of molecules of insulin and of glucose available in the body. Considering the sugar as the substrate on which the insulin acts, we can state that when this is relatively large the amount attacked will be almost directly proportional to the number of units of insulin given ; but, on the other hand, when the amount of the substrate is relatively small the increasing amount of it attacked by each added unit of the insulin will become progressively less and less.

In other words, the reaction must proceed according to a logarithmic curve, like that which Murray Lyon has shown to obtain in the action of epinephrin on the blood pressure, and which is somewhat similar to that of the action of enzymes. Such a curve, expressed in the formula $(u)^{0.85} = 10^{1.86}$, has been plotted by Dr. Frank N. Allan (Fig. 15), and it will be seen that the actual values observed in different animals and represented by the dots and crosses, fall satisfactorily along it.

This close correspondence between the theoretical and the observed values would lead one to expect that insulin could readily be assayed by determining the glucose equivalent of depancreatised animals, by the method just outlined. If this were possible then, instead of expressing the strength of insulin in terms of its power to lower the blood sugar of normal rabbits, it could be done on the much more useful basis of glucose equivalents per unit. A thorough test of the practicability of such a method has been made by Allan (1925), but with results which, when compared with the much simpler method as used at present on rabbits (p. 350), do not justify its adoption.

Glucose equivalents have also been observed by various clinical investigators on diabetic patients, the method usually adopted being somewhat different from the foregoing, in that, after the glucose tolerance has been determined on a certain diet without insulin, the increased tolerance following the administration of insulin is noted, the grammes of glucose accounted for by each unit being calculated by dividing the extra amount metabolised by the number of units given. The same relationship between the amounts of insulin administered and of glucose metabolised is revealed by both methods, although the absolute values for the glucose equivalents are lower by the clinical method, as will be evident from a study of the following figures :—

Number of Units.	Total Glucose Metabolised.	Number of Grammes per Unit (glucose equivalent).	
		1	2
4	82·0	20·5	—
8	110·0	12·5	—
12	111·5	9·3	3·7
16	117·5	7·3	3·0
20	121·5	6·1	2·5
24	125·0	5·2	2·2
28	127·0	4·5	1·9
32	128·5	4·0	1·7
36	130·0	3·6	1·5

The figures in column 1 are obtained by dividing the total glucose metabolised by the number of units given (physiological method), and those in column 2 by dividing the extra sugar metabolised by the extra number of units given, the 82 gms. metabolised with four units being taken as the basis (clinical method).

These differences in the results by the two methods show that the administration of a small dose of insulin given to a completely diabetic dog has a much greater effect than when it is given to a diabetic patient, in whom insulin derived from the pancreas is already present. If conditions could be made strictly comparable, the amount of sugar utilised per unit of insulin would probably be the same in the diabetic dog as in the diabetic man, which means that the insulin requirement of an animal is not affected to any appreciable degree by its size or weight. This conclusion is in accord with the clinical findings of Campbell (1924) and of Joslin, Gray, and Root (1922). There may be another cause for the apparent difference between the glucose equivalents obtained in the laboratory and in the clinic, dependent on the fact that the glucose derived from protein (and fat) is metabolised with greater difficulty than that derived from ingested carbohydrate. Wilder, Boothby, Barborka, Kitchen, and Adams (1922), for example, have noted that insulin given to a diabetic patient, when on a diet containing much protein, caused much less reduction in the glycosuria than when the same dose was given to the same patient on a diet composed largely of carbohydrate, the caloric and the total glucose values of the two diets being kept the same. Thus, while on a diet of 45 gms. protein, 142 gms. fat and 11 gms. carbohydrate (i.e. 150 gms. available glucose and 1956 calories),

15 units of insulin caused the daily sugar excretion of glucose to be reduced from 57·8 to 9·46 gms., giving, therefore, a glucose equivalent of 3·22, whereas while on a diet of 159 gms. protein, 118 gms. fat, and 41 gms. carbohydrate (i.e. 145 gms. available glucose and 1907 calories), 15 units only reduced the sugar excretion from 76·10 to 47·15, giving a glucose equivalent of 1·9. This would seem to indicate that the sugar derived from the body protein (and fat) is not so readily metabolised under the influence of insulin as that absorbed from ingested carbohydrate.

It seems certain that the variable values given by clinical observers for the glucose equivalent of insulin are dependent on the internal secretion from the undamaged islets of Langerhans. The extent to which this is occurring from time to time cannot be the same, so that an uncontrollable variable is introduced which makes it highly unlikely that constant clinical results could be obtained (cf. Campbell, Allen, Williams, Wilder, and Boothby).

The Effect of Insulin on the Glycogen in Diabetic Dogs.—There is no doubt that the observation, originally made by Minkowski, is correct, that no glycogen, or only traces of it, is deposited in the liver of completely depancreatised dogs, even after feeding with large quantities of glucose. There is some doubt as to whether any may be formed when fructose is fed. Minkowski states that this may occur, a conclusion which Cruickshank (1913) was unable to substantiate. We have found that the administration of large quantities of cane sugar for the last three days of life, to an animal depancreatised seven days previously, caused 1·23 per cent. of glycogen to be deposited in the liver; but in another animal similarly treated, and which had been depancreatised for eleven days, only 0·046 could be found. In neither of these animals was the respiratory quotient raised by the administration of the sugar. In no case have we found that the administration of glucose caused any significant quantities of glycogen to be deposited in the liver, as is shown in Table VII.

After the administration of insulin, along with sugar, large quantities of glycogen make their appearance in the liver, as is exemplified in the results in Table VIII.

In the first observation of the same type, made by Collip,

the percentage of glycogen found in the liver after the animal had been given very large quantities of sugar along with insulin was so large that it was difficult to determine it with accuracy; it was apparently over 20 per cent.

TABLE VII.

Date.	Days Depancreatised.	Glycogen Content.	
		Liver.	Heart.
		Per cent.	Per cent.
April 24 (1923)	5	0·09	0·47
May 1 ,,	4	trace	0·38
,, 4 ,,	5	trace	0·12
,, 5 ,,	3	0·06	0·61
,, 7 ,,	4	0·06	0·33
,, 11 ,,	3	0·07	0·65
,, 14 ,,	4	0·03	0·60

TABLE VIII.

Date.	Days Depancreatised.	Days during which Insulin and Sugar were Given.	Per cent. Glycogen in Liver.
Feb. 21 (1922)	7	5 days	12·58
Jan. 14 ,,	4	Less than 1 day	2·70
April 28 ,,	3	2 days	11·40
May 2 ,,	5	1 day	4·80

The importance of the formation of glycogen as the result of the administration of insulin, is undoubtedly very great, and, as has been indicated above, the appearance of the acetone bodies is probably related to the using up of all of this substance, at least in the liver. It is also considered (see Lusk, Verzár) that the ability of the R.Q. to rise when sugar is administered depends on whether or not there is any glycogen in the liver. It is, however, very difficult to make certain that these relationships are as close as they are commonly assumed to be. It will be necessary to have available many more determinations of glycogen in depancreatised dogs than is at present the case before these relationships can be accurately stated.

These results raise the question as to whether insulin can also cause glycogen to be formed in depancreatised animals without giving them sugar. Cori (1923) has contributed two

results which indicate that such is the case. At the time of the pancreatectomy, portions of liver were removed from each animal for glycogen determination. This was also done when the animals were killed four days later, during which time neither animal received any food, but only insulin (during the last two days). The percentages of glycogen at the time of operation were 0·067 and 0·072, and after four days, 2·82 and 1·87 respectively. In an observation on a cat treated with insulin for seven days following pancreatectomy, but also given glucose on account of hypoglycæmic symptoms, 3·57 per cent. of glycogen was found in the liver after death. It would appear from these results that the glycogen can be formed even in the absence of ingested carbohydrate, indicating, therefore, that it must be derived from the sugar which is split off from protein, or derived from fat.

With regard to glycogen in other tissues, we have been unable to detect any significant difference in the amount present in the skeletal muscles of depancreatised animals before and after giving insulin, but there is some evidence to indicate that insulin brings about a reduction in the amount present in the heart. In this connection it is important to remember, as was first shown by Cruickshank (1913), that the diabetic heart contains a distinctly higher percentage of glycogen than the normal one; thus, in the heart of sixteen depancreatised dogs the average was 0·7 per cent., as compared with 0·5 per cent. in observations on six normal animals. We have found 0·79-0·92 per cent. in different portions of the heart of one depancreatised dog which had been fed on sugar without insulin, and 0·98 per cent. in that of another similarly treated animal. In four depancreatised dogs, on the other hand, to which insulin, as well as sugar, had been given, the percentages of glycogen were 0·725, 0·600, 0·570, and 0·296 respectively. We are not quite certain that this effect of insulin on the glycogen of the heart is of significance, since McCormick has found, in untreated, depancreatised dogs, that the glycogen content may not infrequently fall within the normal limits, as is seen to be the case in the results tabulated in Table VII (p. 102). It is possible that these comparatively low percentages of glycogen were dependent on the fact that the animals from which they were obtained were kept under ether for some time before being killed.

The Effect of Insulin on the Fat of the Liver and Blood.—The percentage of fat in the (moist) liver and in the blood is well known to become increased after pancreatectomy, especially when the diabetes has continued for some time. The values shown in Table IX. will serve to illustrate. When insulin is given, this fat invasion of the liver, as well as the lipæmia, becomes much less, and may return to within the normal limits, as is also shown in Table IX.

TABLE IX.

TOTAL FATTY ACIDS IN DEPANCREATISED DOGS, WITH OR WITHOUT INSULIN.

No. of Days after Pancreatectomy.	Total Fatty Acids.		Remarks.
	Liver.*	Blood.†	
	Per cent.	Per cent.	
5	25·3 23·9	1·07 1·08	Not fed sugar, nor given insulin.
5	12·80	1·02	,, ,, ,,
4	12·75	1·13	,, ,, ,,
5	33·0	1·58	,, ,, ,,
3	8·8	0·72	,, ,, ,,
4	23·1	0·74	,, ,, ,,
3	15·6	0·85 0·91	,, ,, ,,
4	26·24	0·76 0·79	,, ,, ,,
6	12·25	—	Fed sugar but insulin not given.
4	14·10	1·21	,, ,, ,,
7	9·90	1·12	,, ,, ,,
6	7·425	0·33	Sugar + insulin 1 day
3	2·190	0·53	,, ,, 2 days
5	4·41	—	,, ,, 1 day
3	10·28	—	,, + overdose insulin ‡
3	26·36	0·37	,, ,, ,,

* Leathes' method. † Bloor's method.

‡ These animals died a few hours after insulin.

No data are available to indicate the time relationship between the changes in concentration of fat in the blood and that in the liver, following the administration of insulin, although, as has already been pointed out (p. 93), the changes in the blood fat occur much more slowly than the changes in ketone bodies, sugar, and phosphoric acid. More work is needed in this field, especially since, in the lipæmia of diabetes mellitus, the effect of insulin is sometimes not readily obtained, especially in severe cases. The clinical experience, even in mild cases, is that the diminution of blood fats under insulin is decidedly a

slow process (cf. Campbell and Macleod). It would be of great importance to obtain more precise data concerning the time relationships in the changes brought about by insulin in the sugar, fat, and ketones of the blood in relationship to the glycogen content of the liver, and the behaviour of R.Q. after sugar.

We will defer till later (p. 125) an account of the effect of insulin on the R.Q. in diabetic animals but it may be mentioned here that Best and Hepburn found at an early stage in the work that when sugar and insulin were given to such animals the quotient rose much more markedly than when sugar alone was given.

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CHAPTER IX.

THE D : N RATIO.

The D : N, or the G : N, ratio expresses the amount of glucose excreted by the urine over a fixed period of time, as compared with that of nitrogen. During starvation, or when the food contains no carbohydrates which can yield reducing sugars on hydrolysis, the ratio is obtained directly from the urinary analysis, but when the food contains carbohydrates the corresponding amount of reducing sugar must be deducted from the glucose excreted before the ratio is calculated.

The value of the ratio depends on its serving as an indicator of the source in the body of the sugar not derived from ingested carbohydrate. When it is determined from day to day in a starving animal made diabetic by frequent administration of phlorhizin, it remains constantly at 3·65, and upon this fact depends the conclusion that 100 gms. of protein (containing 16 gms. nitrogen) in the body can produce 58·4 gms. of glucose. This serves as the basis for the calculation of the amount of sugar which can be derived in the animal body from protein, so that when higher ratios are obtained it is concluded that some of the sugar must be coming from other sources, such as fat. It can be seen, therefore, that determination of the D : N ratio in diabetes is of great importance in tracing the source of the sugar which is excreted ; it furnishes the most important evidence as to whether the source may be fat, as well as protein.

When this evidence is weighed, however, it is, we think, inconclusive in many regards, for although ratios of 3·65 can be obtained in animals poisoned by phlorhizin, this is not always, if indeed usually, the case, either in animals after pancreatectomy (p. 44) or in the clinical forms of diabetes. In the former, when the animals are starved, the ratio is seldom higher than 2·8, and it does not usually remain constant from day to day; and, in the latter, ratios considerably greater than 3·65 have not infre-

quently been observed. Many of these high ratios in man have, however, been obtained when the patients were on mixed diets—the necessary allowance being, of course, made for preformed carbohydrate—and the question arises, as to whether an animal under phlorhizin, and receiving no carbohydrate with the food, is to be considered as comparable with one in which these foodstuffs are abundant, especially since, as is well known, carbohydrates exercise a profound influence on the process of metabolism, not only of protein itself, but also of fat.

In accepting a ratio of 3·65 as indicating the maximal production of sugar from protein in the body, it is necessary to assume, either that none of this sugar is oxidised in the body, or that only a constant proportion of it is oxidised, and also that the sugar and the nitrogen derived from protein are excreted from the body at the same rates. When carbohydrates are present in the food another source of error is incurred on account of no allowance being made, in subtracting the ingested sugar from that excreted, for the possible destruction of sugar by micro-organisms in the digestive tract. When this occurs more sugar is deducted than ought to be, so that the ratio is too low.

In the present chapter [1] we will review some of the more important contributions which have been made with regard to the ratio in the three varieties of diabetes—pancreatic, phlorhizin, and clinical—and then proceed to see in how far the results justify the commonly accepted belief that protein, but not fatty acid, can serve as a source of sugar in the animal body.

1. Pancreatic Diabetes.—It will be recalled that Minkowski found that the average D : N ratio obtained on seven meat-fed depancreatised dogs on twenty-two different days was 2·8 (the lowest being 2·62 and the highest 3·05). Sometimes in starved animals, also, ratios approaching this value were obtained. Higher ratios were noted during the first few days following the operation, and lower ones during the period immediately preceding death. The cause for the high ratios in the first few days following the operation was, no doubt, that the glycogen reserves were being gradually converted into glucose, and evidence that this was occurring was furnished by the fact that during these days the urinary excretion of nitrogen was steadily rising. The

[1] I wish to acknowledge the free use which I have made in this chapter of the admirable monographs by Graham Lusk and Geelmuyden.

cause for the falling off of the ratios in the few days preceding death cannot be given, although it is significant that during this period the previously excessive excretion of nitrogen was also steadily falling.

Other observers have usually found the ratios in completely depancreatised dogs somewhat lower than 2·8 (cf. Pflüger, 1905 ; and Embden and Salomon, 1905). After partial removal of the pancreas they may be very low for some time after the operation, and then gradually rise to about 2·8, as the remaining portion of the gland becomes atrophied (Sandemeyer). Langfeldt states that the ratio in these cases may ultimately rise to between 4·21 and 4·35. Variable ratios are reported by F. M. Allen in similar observations.

A factor which may have considerable influence on the ratio in recently depancreatised animals is the generally poor condition into which they are brought by the severe operation, quite apart from the diabetic state. In order to avoid this possible source of error we have repeated the observations on the D : N ratio of dogs which were in good condition as a result of treatment with insulin for some weeks following the pancreatectomy. The ratio was then determined for periods of a day each at varying intervals after discontinuing the insulin, no food being meanwhile given. The following results, from analyses made by Markowitz, are instructive :—

Dog A.—Large mongrel. Time of pancreatectomy unrecorded. Insulin and food withdrawn 13th November. Blood sugars also determined daily.

Urine of Nov.		14-15	Glucose	45·96	Nitrogen	5·10	D : N	9.01	B.S.[1] 0·410
,,	,,	15-16	,,	15·86	,,	4·61	,,	3·44	,, —
,,	,,	16-17	,,	21·42	,,	6·29	,,	3·4	,, 0·333
,,	,,	17-18	,,	20·02	,,	6·42	,,	3·12	,, 0·312
,,	,,	18-19	,,	17·16	,,	5·92	,,	2·90	,, 0·357
,,	,,	19-20	,,	14·9	,,	5·66	,,	2·63	,, 0·308

Dog Mo.—Mongrel. Date of pancreatectomy unrecorded. Insulin and food withheld 7th January, and urine collected for next two days and then for three days a week later, during which time no food was given.

Urine of Jan.		8-9	Glucose	10·56	Nitrogen	5·21	D : N	2·03
,,	,,	9-10	,,	0·52	,,	3·49	,,	—
,,	,,	17-18	,,	2·6	,,	1·53	,,	1·41
,,	,,	18-19	,,	2·64	,,	1·59	,,	1·65
,,	,,	19-20	,,	1·01	,,	1·71	,,	1·12

[1] B.S. = blood sugar.

Dog M.—Small, lean fox-terrier. Depancreatised 22nd January. Insulin and food withdrawn 12th March, and urine removed by catheter.

Urine of Mar.	12-13	Glucose	15·5	Nitrogen	1·37	D : N	11·3
,, ,,	13-14	,,	18·1	,,	1·37	,,	13·2
,, ,,	14-15	,,	7·5	,,	1·56	,,	4·8
,, ,,	15-16	,,	4·23	,,	1·90	,,	2·23
,, ,,	16-17	,,	5·01	,,	2·38	,,	2·10
,, ,,	17-18	,,	5·82	,,	3·03	,,	1·96

Dog L.—Lean mongrel. Depancreatised February. Insulin and food withdrawn 17th March, but no urine collected until three days later.

Urine of Mar.	19-20	Glucose	7·75	Nitrogen	2·89	D : N	2·79
,, ,,	22-23	,,	6·83	,,	3·48	,,	1·91

Dog P.—Lean Boston bull. Depancreatised 26th February. Insulin and food withdrawn 11th March, but urine not collected till next day.

Urine of Mar.	12-13	Glucose	14·0	Nitrogen	2·76	D : N	5·08
,, ,,	14-15	,,	4·41	,,	3·08	,,	1·43
,, ,,	15-16	,,	2·62	,,	2·88	,,	0·91

Ratios approaching 2·8 were obtained only in the first animal (A) from the fourth to the sixth day after removal of insulin. In the others it was consistently lower except during the days immediately following withdrawal of insulin, when it might stand at any level, dependent no doubt on the amount of glycogen stored in the liver, as a result of the previous treatment with insulin.

It may be remarked that all of these animals were in excellent condition when the insulin was discontinued—usually lean—and that general symptoms of varying severity appeared as described on page 88. All of them were completely depancreatised, as judged by careful post-mortem examination, and in the cases of dogs M, L, and P, by finding that the administration of glucose (40 gms.), by mouth, did not have any effect on the respiratory quotient (see also p. 125). We obtained no evidence for the commonly accepted belief that a D : N ratio of 2·8 is characteristic of pancreatic diabetes, at least during starvation, although the work of others would seem to indicate that it may remain at about this level during feeding with meat.

2. Phlorhizin Diabetes.—The observations in this form of experimental diabetes have been more numerous than in that due to pancreatectomy, partly because the diabetic condition is more conveniently induced, and partly because the observations need not be restricted to the dog. In the earlier of them, the ratios obtained were by no means constant, because insufficient

regard was taken of the influence of the carbohydrate stored in the body on the excretion of glucose during the first day or so following the giving of the drug. This source of error comes into play, not only during the first day or two after starting the injections, but also when the phlorhizin is not given with sufficient frequency to maintain a constant diabetic condition. In the latter case, intervals of recession of the diabetic state will allow carbohydrate to become stored again in the body, thus causing abnormally high excretion of glucose to occur during the next diabetic period. Lusk has shown that the action of phlorhizin, given subcutaneously, may not last for more than seven hours in rabbits, and probably for about the same time in dogs, especially when the drug is given dissolved in alkali. When it is given in finely powdered form suspended in oil, as suggested by Coolen, the duration of action is prolonged to several days. The usual practice, following the recommendation of Lusk, is to inject phlorhizin, dissolved in carbonate solution three times daily, the dose being usually 1 gm., in such animals as the rabbit and cat, and 2 gms. in dogs of average size.

For cats, rabbits and sheep under the complete influence of phlorhizin, the D : N ratio has been found by numerous investigators to be about the same as that for depancreatised animals while fed on meat, namely, about 2·8. This is shown in the following table :—

TABLE X.

RATIOS IN DIABETES OF D : N : : 2·8 : 1.

Day.	Dog. Pancreas Diabetes.	Dog. Phlorhizin and Camphor.	Cat. Phlorhizin.	Goat. Phlorhizin.	Rabbit. Phlorhizin.
2nd day of diabetes	—	—	—	2·95	2·89
3rd ,, ,,	2·88	—	2·93	2·90	2·69
4th ,, ,,	2·94	—	2·80	2·78	—
5th ,, ,,	3·09	—	2·93	—	—
Day unknown	—	2·8	—	—	—

(*From* LUSK.)

In dogs, on the other hand, Reilly, Nolan, and Lusk (1898) found the average ratio to be 3·75. It may vary slightly for different dogs, so that when it is used for purposes of calculating

the sources of sugar, as, for example, following the giving of some possible sugar-producing foodstuff, it must be determined for each animal on which the observation is to be made. In twelve out of fifteen dogs given phlorhizin, the ratio lay between 3·40 and 3·89, irrespective of whether the animal was starved or was fed on protein, or protein and fat. When protein is given to a previously starved animal, however, there may be a short period during which the D : N ratio becomes lowered, which may be accounted for as being due to the fact that excretion of sugar is slower than that of nitrogen.

As in pancreatic, so also in phlorhizin diabetes, the excretion of nitrogen is much higher than normal; thus in the rabbit it has been found to be raised 167 per cent., and in the goat 238 per cent. above the normal, the maximum being reached in two to three days after commencing administration. It then falls gradually as the condition progresses, this being accompanied by a decline in body weight. Accompanying the increase in the excretion of nitrogen there occurs also an increase in that of phosphorus (see also p. 332).

A considerable amount of attention has been given to the effect produced on the D : N ratio of phlorhizined animals by adding fat to a diet previously consisting of protein alone. In the earliest observations (by von Mering) the ratio was seen to rise, the explanation given being that the fat had suppressed the nitrogen excretion. Hartogh and Schumm (1901) reported very high ratios (10·7 and 13·0) as a result of fat-feeding, but the results have been categorically denied by Lusk who, with Mandel, kept a dog constantly injected with phlorhizin in a respiration apparatus and examined the urine at regular intervals. During hunger, 51 gms. of sugar were excreted, the D : N ratio was 3·57 and the respiratory analysis showed that 54·3 gms. of carbon had been oxidised as fat. The animal was then given 100 gms. of lard (Speck) (76·5 gms. carbon) and, as a result, 46·3 gms. of sugar were excreted, the D : N ratio was 3·61 and the amount of carbon oxidised as fat was 53·2. This experiment, as well as others by Loewi, indicates that the addition of fat to the diet has no influence on the excretion of sugar in phlorhizin diabetes. The results would be of much greater value had the ratios observed on fat animals been compared with those of thin ones.

Very exact determinations of the ratio in phlorhizin diabetes in dogs have also been made by Janney and his co-workers (1915). For starving animals the average of a large number of observations by themselves and others was 3·43 which became

3·6 by allowing for the nitrogen of creatine and creatinine and of the purine bases. He also gives the ratios obtained when various forms of flesh were fed to the phlorhizin animals, and the results of these experiments are shown in the following table :—

	Animals from which Flesh was Obtained.				
	Man.	Dog.	Rabbit.	Ox.	Chicken.
D : N ratio . . .	3·6	3·6	3·8	3·6	3·4
Glucose per 100 gms. of protein metabolised . . .	58	58	60	58	54

(*From* LUSK.)

In earlier observations Janney (1915) had shown the percentage amount of glucose derivable from various proteins to be as follows : Casein, 48 ; ovalbumin, 54 ; serum albumin, 55 ; gelatin, 65 ; fibrin, 53 ; edestin, 65 ; gliadin, 80 ; and zein, 53.

The evidence furnished by these observations would seem to show that all the sugar excreted by phlorhizined dogs, both during starvation and during feeding with protein and fat, can be accounted for as being derived exclusively from the non-nitrogenous portion of the protein molecule. A similar ratio has also been observed by S. R. Benedict as a result of the administration of phlorhizin to a man, a patient suffering from cancer.

There are, however, certain observations, especially those of Pflüger and Junkersdorf (1910), which are considered by these authors to indicate that much of the sugar must come from fat. The method of calculating the D : N ratio was, however, entirely different in this case. The exact experiment was as follows : A dog was starved for thirteen days, and from the eighth to tenth day it excreted 48·24 gms. nitrogen. From the eleventh to thirteenth day it was given phlorhizin and fed with fat, and it excreted 133·5 gms. sugar and 55·02 gms. nitrogen. The difference between the nitrogen excretion of the two periods, namely 6·78 gms., divided into the sugar excreted during this period, gives a D : N ratio of 19·7. By similar methods, three other animals gave quotients of 14·6, 7·4, and 7·0 respectively. After Pflüger's death, in a continuation of these observations, Junkersdorf found that although he could not duplicate the above results in lean animals, this was possible in a very fat one, a quotient of 33·98 being obtained by the above method of calculation. The objection to this method of calculating the D : N ratio is that it assumes that all the sugar comes from the extra protein broken down during the period of phlorhizin poisoning,

no allowance being made for that metabolised independently of the phlorhizin. As a matter of fact, when the usual method of calculating the D : N ratio is applied to the published data a value of 2·46 is obtained.

3. Diabetes Mellitus.—It is impossible here to review all the observations in this form of diabetes, but some of the most significant of them may be briefly summarised. Many, particularly those from German clinics, have been made on patients receiving some carbohydrate in their food, allowance for this being given in computing the D : N ratio. The number of observations on patients during starvation, or on an exclusive protein diet, is not large, and few have been published since the modern methods of treatment have come into practice.

Rumpf found an average D : N ratio of 10·0 for a period of fifteen days in the case of a patient allowed some carbohydrate. He stated that the introduction of this foodstuff causes the excretion of nitrogen to increase, indicating an absence of protein-sparing action (1899). In a later publication, ratios of from 0·003-6·8 in six cases of diabetes are reported. In one of these the highest ratio occurred in the first eight days of the observation, when 27-51 gms. of carbohydrate was given, falling during the next four days when this was withheld, to rise again during the last three days of the observation in which the food again included carbohydrate (1902).

From data published by Rosenquist on two diabetic patients kept on a mixed diet containing 50-70 gms. carbohydrate, Geelmuyden calculated an average D : N ratio in one of them, for a period of seventeen days, to be 4·76, and in another for six days, to be 5·26. The latter also reports observations by Mohr, Hesse, and Grafe, and Wolf, and Lüthje. Mohr found an average D : N ratio of 5·96 for a period of eight days in one patient, whose diet contained 4·44 gms. carbohydrate daily. In another, observed during a period of twelve days, it averaged 8·14, when 76 gms. carbohydrate was given. During a later period of eleven days, when the carbohydrate in the food was reduced to 5 gms., the ratio fell gradually to 3·7, and gave an average of 6·6. Hesse observed two patients, one of them a young girl thirteen to fifteen years of age, and the other a man of fifty-seven years. No carbohydrate was contained in the diet of the girl, and the average D : N ratio during the last four days of the observation was 7·53. The diet of the man contained 33·35 gms. carbohydrate, and the average D : N ratio for the last four days of the observation was 10·67. Grafe and Wolf, in one of three carefully observed cases, obtained an average D : N ratio of 5·22 on a diet containing much fat with only small quantities of carbohydrate. This case was conspicuous by there being an excessive loss of nitrogen from the body and marked acidosis. In the other cases reported by these workers the carbohydrate content of the diet varied

considerably from day to day, as also did the D : N ratio, which, however, averaged 6·1. In this case, there was retention of nitrogen. Lüthje recorded D : N ratios varying between 2·5 and 5·7, the highest values being usually observed when there was a loss of nitrogen from the body. The addition of carbohydrate to the diet did not affect the average (after making allowance for the ingested carbohydrate).

It is pointed out by Geelmuyden that abnormally high ratios occur more frequently when carbohydrate is contained in the diet than when the patients are starved or are kept exclusively on protein, and that they may occur either when there is retention of nitrogen (positive nitrogen balance), or when there is nitrogen loss (negative nitrogen balance).

To illustrate this, the observations of Falta and his collaborators are quoted, in which, instead of attempting to determine the D : N ratio on a constant diet, patients were maintained on a standard one to which known quantities of other food principles were superadded. The new D : N ratios were often found to be greater than could be accounted for by supposing that all the extra glucose had been derived from protein. Such cases occurred, especially when excess of protein was fed, and were called "protein sensitive." High D : N ratios in other cases were more readily obtained when carbohydrate was superadded, and these have been called "carbohydrate sensitive." In one patient, for example, it was found that addition of 80 gms. of casein caused an extra 50 gms. of glucose to be excreted in the next three days, whereas with 100 gms. of wheat bread, the glucose excretion was only increased by 6·8 gms. A conspicuous feature of this case was the marked positive nitrogen balance. That these results were not accidental was indicated by the fact that the patient showed practically the same response to protein and carbohydrate feeding one year later. Falta and Gigon showed, by recalculation of results published by Allard, that the D : N ratio was very constant on hunger days, but that it varied greatly when much nitrogen was given, being highest in the urine excreted in the second part of the night. The addition of fat to the diet lowered the excretion of nitrogen somewhat, but caused a greater increase in that of sugar, so that D : N became 10·2. This they consider as a case in which gluconeogenesis was particularly prone to occur from fat, and the fact that the high ratios were liable to occur in the second part of the night is considered significant, since this is the period, according to Magnus Levy, during which the fat of the food is being assimilated.

In contrast to these observations from European clinics are to be placed those of the American school, in which high ratios have not been observed. Mandel and Lusk, for example, describe a case in which the D : N ratio was 3·65-1, corresponding,

therefore, to that observed in phlorhizin animals. This patient was greatly emaciated, and there was a negative nitrogen balance, amounting to 14 gms. in the day. Similar ratios were obtained by Greenwald (1914), and in a case, reported by Geyelin and Du Bois, the ratio of the preliminary fast was 2·95, but later, when the diet contained 100 gms. protein, it rose to 3·97, 4·01, and 3·87 on three successive days. H. O. Mosenthal obtained D : N ratios varying between 2·89 and 3·71 on a patient who was almost fasting (one egg and green vegetables containing 2-6 gms. carbohydrate per day); Janney, one of 3·4; and Allen and Du Bois, one over 3·65. The last mentioned observation was made on a case following a period during which some carbohydrate had been given to prevent coma, so that it is suggested that the high ratio may have depended on the production of sugar out of glycogen stored in the body.

The American investigators are unanimously of the opinion that there must have been some error in the European observations, due possibly to the great difficulty of preventing diabetic patients from obtaining carbohydrate food surreptitiously, or to too brief periods of observation.

The practice of studying only those cases in which the diet is entirely free of carbohydrate does not, however, seem to be entirely free from objection, since it is quite conceivable that this foodstuff may have a considerable influence on the nature of the metabolic process. In this connection it is interesting to note that Geelmuyden, in recalculating the D : N ratios from data published by Benedict and Joslin (1912), found ratios that were frequently greater than 3·65. Since these patients were receiving small quantities of carbohydrate, it is unlikely that excessive breakdown of glycogen could be held accountable for the relatively high excretion of sugar.

Taking these results as a whole, it can be seen that the D : N ratio in human diabetes, even when the conditions are strictly controlled, are by no means constant. That is what one would expect, since it can seldom, if ever, be the case that the pancreas in diabetes mellitus becomes so far destroyed by disease that no internal secretion of insulin is possible. So long as any of this endogenous secretion remains, there may be periods during which carbohydrate is stored in the body as glycogen, and others in which these stores become broken down

into sugar, thus accounting for the variability in the D : N ratio. There is evidence that in the normal animal the internal secretion of insulin varies from time to time (p. 289), and this is also likely to be the case in diabetes, although only a remnant of functioning pancreas remains.

Highest Ratio Possible when the Sugar comes from Protein.—In discussing the above cases, the assumption has been made that when the D : N ratio exceeds a certain value it indicates sugar formation from fat. Various methods have been used to calculate the highest limit to which the ratio could rise were protein exclusively the source of the sugar.

Von Mering assumed that 135 gms. protein might give a ratio of 8, but this is undoubtedly too high. Falta calculates as follows : Assuming that muscle protein contains 16·65 per cent. N + 52·38 per cent. C. (Zuntz), and that 16·65 gms. N. require 8·32 gms. C. to form urea, there are 44·06 gms. C. available to form 110·15 gms. glucose, so that the D : N ratio is 6·62. On this basis, however, the energy balance does not come out properly. Thus,

110·15 gms. D. = 413·06 Cal. and
16·65 gms. N. (as urea) = 90·41 Cal. (1 gm. N. = 5·43 C. as urea)

so that 503·47 Cal.

leave the body unused as sugar and urea. Since the energy value of 100 gms. pure protein is 540 Cal., 36·5 Cal. is unaccounted for, which, Falta thinks, may be used for the swelling and solution of protein.

More probably correct is the ratio calculated by Rubner, who subtracts the specific dynamic value of 1 gm. nitrogen from the total calorie value; 1 gm. of nitrogen from muscle protein yields, by combustion in the body, as total energy 26·0 C., subtracting from this the specific dynamic value of 7·4 C. gives a true energy value from 1 gm. nitrogen of 18·6. This assumes, of course, that all of the 7·4 C. comes from protein itself. The D : N ratio is, therefore : $\frac{18{\cdot}6}{3{\cdot}743} = 4{\cdot}97$.

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CHAPTER X.

THE RESPIRATORY QUOTIENT.

The Respiratory Quotient (R.Q.) expresses the relationship between the amount of oxygen retained in the body and that of carbon dioxide, which is meanwhile eliminated. Since it must be the same at whatever rate complete combustion occurs, the quotient for each of the proximate principles of food can be computed from their chemical formulæ; thus, it is unity (1·0) for carbohydrate, 0·707 for fat, and 0·809 for protein. It is therefore a valuable indicator of the kind of food being oxidised in the body, and it is theoretically possible, by also considering the excretion of nitrogen and the total O_2 intake, to calculate the number of grammes of each foodstuff undergoing metabolism (cf. tables of Zuntz, Lusk, and Rubner). The metabolism of carbohydrate is imperfect in the completely diabetic animal, so that it is impossible, in them, for the quotient to stand higher than that of protein (0·8), and, indeed, since the energy requirements cannot be adequately covered by this foodstuff alone, some fat must also be used, so that the quotient is actually somewhere between 0·7 and 0·8. Since similar quotients are common in the post-absorptive state in the normal animal, its actual level is of no value as an aid to the diagnosis of diabetes, but its behaviour following the ingestion of carbohydrate is useful for this purpose: it rises promptly in the normal animal, but fails to do so, or may even decline, in the diabetic. Examples of its use in this connection are shown in Table XI.

There are, however, a number of factors which may come into play in the body to modify the quotient. In the first place, intermediary products of metabolism may fail to be completely oxidised within the time during which the quotient is being measured. Sugar, for example, may become converted into fat, involving thereby a reduction process, by which the excess of oxygen is eliminated as CO_2, so that the quotient is

raised to over 1·0. This occurs in hibernating animals during the period in autumn when they are feeding on nuts and other foods rich in carbohydrate. On the other hand, the oxidation of fat may not proceed to completion, so that incompletely oxidised substances, such as the ketone (acetone) bodies, are excreted, with the result that the quotient falls, because the intake of oxygen is increased out of proportion to the CO_2 eliminated. A similar lowering of the quotient must also occur when fat or protein is converted into carbohydrate, and herein lies one of the chief interests in the determination of the quotient in diabetes. The amount of sugar coming from protein, as we have seen, can be computed from the D : N ratio (p. 106), so that, if the respiratory quotient should be lower than the lowest possible for fat alone, and this cannot be accounted for by the amount of ketone bodies meanwhile formed, strong evidence is furnished that some of the sugar is being derived from fat.

Based on these theoretical considerations, Lusk, Magnus Levy, and, more recently, Geelmuyden, have calculated what the quotient would be under standard conditions in the diabetic animal. Geelmuyden calculates as follows : Assuming that fat has the composition C. 76·5 ; H. 11·9 ; O_2 11·5 ; and that the carbon residue of 100 gms. protein, after subtraction for the loss in the urine and fæces, contains 41·5 gms. carbon, 4·4 gms. hydrogen, and 7·69 gms. oxygen—with the corresponding urinary nitrogen of 16·28 gms.—then, in a case of diabetes in which the diet contains 100 gms. protein and 230 gms. fat with a total energy value of 2549 calories and a D : N ratio of 3 (that is, 48·84 gms. sugar), the R.Q. is as follows :—

N.-free residue from 100 gms. protein =	41·50 g. C.	4·40 g. H.	7·69 g. O.
230 gms. fat	+175·95 g. C.	+ 27·37 g. H.	+ 26·45 g. O.
	217·45 g. C.	31·77 g. H.	34·14 g. O.
− 48·84 gms. sugar . . .	19·54 g. C.	3·26 g. H.	26·05 g. O.
	197·91 g. C.	28·51 g. H.	8·09 g. O.
48·84 gms. sugar is isodynamic with 19·64 gms. fat . . .	+ 15·02 g. C.	+ 2·34 g. H.	+ 2·26 g. O.
	212·93 g. C.	30·85 g. H.	10·35 g. O.
O_2 necessary for combustion .	567·813 g.	246·80 g.	

$$\frac{CO_2}{O_2} = \frac{567{\cdot}813}{567{\cdot}813 + 246{\cdot}80 - 10{\cdot}35} = \frac{567{\cdot}813}{804{\cdot}26} = 0{\cdot}706.$$

When allowance is also made for 40 gms. of oxy-butyric acid, the above quotient is only lowered to 0·694. Indeed, as Magnus Levy first pointed out, the excretion of this amount of oxybutyric acid, which is about the

maximal, only lowers the quotient by 0·012 for all possible combinations of fat and protein in the diet. By this method, Geelmuyden has calculated the possible R.Qs. for diets all yielding 2550 calories per day for a man weighing 70 kg. with different proportions of protein and fat and varying D : N ratios. The table shows that the D : N ratio has a far greater influence on the R.Q. than has the proportion of protein and fat, or the excretion of β-oxybutyric acid. For example, with a diet of 200 gms. protein and 186 gms. fat and a D : N ratio of 6·37, R.Q., without allowing for oxybutric acid, equals 0·662. Under the same conditions, but with a D : N ratio of 3, the R.Q. equals 0·703. We may take these figures to represent the extremes which are possible during gluconeogenesis from protein.

A simpler method of calculation, based also on the original one of Magnus Levy, is given by Lusk as follows : When the D : N ratio is 3·65

	O_2 gms.	CO_2 gms.
Normal oxidation of 100 gms. beef protein	138·18	152·17
Deduction for 16·28 gms. × 3·65, which corresponds to 59·41 gms. glucose .	63·38	87·15
	74·80	65·02

Converting the ratio of weights into the ratio of volumes, one finds that the diabetic R.Q. for protein is 0·632.

Before proceeding to see whether the quotients actually observed in the various forms of diabetes are below those theoretically possible, without assuming any conversion of fat into carbohydrate, it will be well to consider several conditions which must be fulfilled in determining them.

In the first place, the measurement of the respiratory exchange must be made over a period of time which is of sufficient length so that the combustion process in the body is completed and conditions temporarily affecting the excretion of carbon dioxide have been compensated for, such as changes in breathing due to nervous or chemical stimulation of the respiratory centre. It is useless, for example, to compute the quotient for brief periods of time (twenty to thirty minutes), since disturbances in breathing (due to a mask, for example) may alter greatly the excretion of CO_2 without influencing the intake of oxygen. But even if this source of error be eliminated, either by using a respiratory cabinet in place of a mask, or by making the observations on patients who are accustomed to the latter, such brief periods of observation are not dependable. They should not be shorter than those required for the elimination of all the nitrogen, sugar, and β-oxybutyric acid derived from proteins and fats. Thus,

when sugar is formed out of protein in the diabetic organism, this may be excreted before the nitrogen, and in any case both of these are excreted some time after the respiratory exchange accompanying the gluconeogenic process is completed. When to these difficulties are added the technical ones met with in the measurement of the oxygen retention, it is evident that there can be but few of the published results, of short period observations, that are of much value.

As can be seen from Geelmuyden's calculations, a respiratory quotient of 0·65 or less, with a D : N ratio of 6·37, cannot be interpreted in any other way than by supposing that sugar is formed from fat. It is important therefore to consider those cases in which quotients of about this magnitude have been reported.

The Quotient in Diabetes Mellitus.—The following are quoted mainly from Geelmuyden's monograph : Probably the most complete observation, and also the oldest on record, is that of Pettenkofer and Voit (1867). The patient received carbohydrate in the food, and on one day the D : N ratio was 5·15 and R.Q. 0·664. Objection has been taken to the results on the score that the oxygen was not measured directly, but by the difference in weight of the patient and the absorption tubes. The results of Grafe and Wolf, of Nehring and Schmoll, and of Weintraud and Laves are not considered reliable because of faulty methods of technique, or because the periods of observation were too brief. These possible sources of error are largely eliminated in the observations of Benedict and Joslin, in which the periods were of one hour duration, and were made in the morning before any food had been taken.[1] In certain of the patients kept on a very low diet, very low R.Qs. were observed, between 0·61 and 0·66. In a later publication, the effects of food were investigated, and in one patient (P) the R.Q. was found to sink, after ingestion of protein and fat, from 0·713-0·61. In yet another patient (R) the quotient sank from 0·73-0·67 after taking carbohydrate. Benedict and Joslin, however, do not consider this short-period quotient of much value as an index of the type of metabolism.

Mohr (1907) observed in two patients, one of them a woman with mild diabetes and the other a child with severe diabetes, that ingestion of protein caused the intake of oxygen to become increased without any change in the excretion of CO_2, giving an R.Q. which sometimes went below 0·65.

[1] These investigations were conducted primarily for the purpose of determining whether there was any change occurring from the normal in the energy expenditure of diabetic patients and for this purpose the one-hourly periods were sufficiently long.

Rolly (1912), using the Zuntz-Geppert apparatus, has made the interesting observation that severe cases, during the first few days after being admitted to the hospital, show, in the post-absorptive condition in the morning, relatively high values for oxygen intake. This author also observed that ingestion of carbohydrate sometimes caused a slight falling in the R.Q. in severe cases.

Bernstein and Falta (1918) have drawn the interesting conclusion that the ingestion of carbohydrate raises the R.Q. in normal persons only after the glycogen stores have become more or less filled. Ingestion of sugar, for example, had only a very delayed effect on the R.Q. in the case of persons in whom the glycogen stores had been depleted by starvation and muscular exercise. In contrast to this, the intravenous injection of carbohydrate immediately caused the R.Q. to rise. In mild diabetes it often requires three days for the R.Q. to respond after the ingestion of carbohydrate, such as oatmeal or fructose, and in severe cases it remains unchanged. After intravenous injection of sugar in a severe case of diabetes the quotient actually fell, this being accompanied by the reappearance of all the injected sugar in the urine, along with a surplus of sugar derived from the organism. An interesting observation is recorded of the effect of epinephrine in diabetes : the R.Q. fell from 0·611-0·604, the sugar excretion meanwhile becoming much increased. These results can be interpreted by assuming, either that sugar was derived from fat or that some glycogen had been stored in the liver and then broken down by the action of the epinephrin.

Further observations are those by Leo (1891), Magnus Levy, and Leimdörfer (1912). The first of these investigators did not observe subnormal quotients. Magnus Levy's observations were made under basal conditions, and lasted from forty to fifty minutes. In three cases of severe diabetes, in highly emaciated patients, the lowest R.Q. was 0·637, and in three milder cases, each of which was corpulent, a quotient of 0·640 was observed. Although these quotients would seem to indicate gluconeogenesis from fat, Magnus Levy does not put this interpretation upon them. Leimdörfer also determined the quotient under basal conditions on seven patients (five severe and two mild), kept either on carbohydrate-free diets or on diets containing vegetables and oat meal. Quotients falling between 0·638 and 0·678 were found in the severe cases on strict diet, but occasionally lower ones than 0·600 were observed to occur.

In reviewing the foregoing cases, Geelmuyden points out that it is unlikely that the low R.Q. can, in all cases, have been due to the faulty conditions of investigation. The considerable variations which have been noted to occur, as, for example, in the observations of Benedict and Joslin, may in themselves be significant features of the disease. No two patients will necessarily react in the same way, either to starvation or to the

ingestion of fat. This author also recommends, in the further investigation of this problem, that respiratory observations ought to be made before, as well as after, treatment of the case by dietetic measures, because the metabolic processes may be considerably affected by the presence of preformed carbohydrate in the food, as is evidenced by the fact that its administration to a diabetic animal may cause more sugar to be excreted than can be accounted for by the added carbohydrate *plus* the sugar coming from protein, as determined by the D : N ratio (p. 116).

Graham Lusk, who may be regarded the leader of the American school, does not consider that the "non-protein respiratory quotient" in severe diabetes indicates gluconeogenesis from fat. With a diabetic patient having a D : N ratio of 3·97, this quotient worked out at 0·699, although 71 gms. of β-oxybutyric acid were excreted on the day of the observation. Since this would tend in itself to lower the quotient, it is concluded that the approximate R.Q. of fat was really attained. In another case, with a D : N ratio of 3·5, the quotient was 0·7. Lusk points out, however, that many secondary factors may enter in to complicate the quotient, such, for example, as the ammonia used to neutralise butyric acid, removing CO_2 from its normal synthesis into urea, and thus causing it to be eliminated in the respired air. Lusk says that "the actual finding of the respiratory measurement carried a refutation of the idea that fat may be converted into sugar," and later, "one by one the bulwarks of the doctrine of the conversion of fat into glucose have been shattered, and it may now be relegated to the realm of scientific superstition." Taking the foregoing results as a whole, as evidence for or against the hypothesis that sugar can be formed out of fat in diabetes, it is evident, however, that a verdict of "not proven" is the best that can be arrived at. Further investigation under strictly controlled conditions is called for, and since there is evidence that carbohydrate may lower the quotient in diabetes, the observations should be made with this foodstuff as a part of the diet.

The Quotient in Depancreatised Animals.—Turning now to the *experimental forms of diabetes*, even fewer results can be quoted in which the above conditions are fulfilled (sufficiently long periods of observation, D : N ratios, and faultless technique of respiratory analysis). In depancreatised dogs, either in a

starving condition or fed exclusively on protein and fat, quotients of 0·71 have not uncommonly been observed (cf. Geelmuyden), but rarely have they been sufficiently below this level to indicate gluconeogenesis out of fat. The quotients of 0·637, reported by Mohr, are not acceptable, because no D : N ratios are given, and, as we have seen elsewhere, these are seldom high in the starving condition in such animals. La Franca reports quotients below 0·65 in depancreatised dogs fed with milk, and although here also no D : N ratios are recorded, it is possible, on account of the ingestion of milk sugar, that there may have been a negative carbohydrate balance (p. 114), and consequently a high D : N ratio.

No doubt one of the difficulties that has stood in the way of prolonged respiratory observations after pancreatectomy has been the poor general condition of the animal. We have therefore repeated the observations, using depancreatised animals kept on insulin until they had completely recovered from the immediate effects of the operation. The insulin was discontinued a few days before making the observations, and most of the animals were also deprived of food, except that glucose was given on certain days. Although these investigations are not yet completed, brief reference to some of the results may not be out of place. A respiratory cabinet constructed according to F. G. Benedict's directions has been used, its accuracy being tested from time to time by burning a weighed quantity of ether in it.[1] Quotients varying between 0·60 and 0·66 were obtained.

Satisfactory evidence of the dependability of the results is also furnished by their constancy. In the usual routine, the animal is placed in the cabinet and the pump allowed to run, with the absorption bottles in circuit, for one full hour before any observation is attempted. In the first actual observation (second hour) it is usually found that quotients decidedly lower than those of subsequent hour periods are obtained. These are not, as a rule, considered. Samples of air are also removed from the chamber and analysed for CO_2 and O_2 at frequent intervals, and if any variation from the usual values are observed to occur, the observation is discarded. From 0·5-0·7 per cent. is considered as the permissible limits for CO_2, and since this level is attained by the second hour at least and remains steady thereafter, no correction

[1] This test is by no means an easy one to carry out with a small chamber (150 litres capacity) because of the difficulty of reducing the size of the flame sufficiently so as to avoid excessive heating of the air and at the same time bring about a rate of combustion comparable with that of a dog of average size.

is made for it in computing the results (those of the first recorded period not being included in doing this). The total duration of each experiment is usually eight hours, urine being removed by catheter just before placing the animal in the chamber, and any voided while in it collected in a suitable tray. It may be added that the cabinet, which is double-walled and packed with cork, is kept in a small room of reasonably constant temperature, and that the movements of the animal are recorded on a revolving drum.

The animals so far used have been some of those depancreatised by Markowitz, who also made the urinary analyses. The respiratory analyses were made by Miss N. R. Hearn and Mr. F. L. Robinson.

Since one object of the observations was to determine the behaviour of the R.Q. after the ingestion of carbohydrate, 40 gms. of a good commercial preparation of glucose (bactodextrose) was usually given at midday, and one hour then allowed to elapse before again measuring the respiratory exchange, the animal being meanwhile in the cabinet with the absorption tubes in circuit.

The results of technically flawless observations are given in Table XI. The animal was usually placed in the cabinet at 9 A.M., and removed at 4 P.M., each day, and the calories per hour were computed, from the average results of the entire period, except when glucose was given, when those of the two last morning hours were taken for the A.M. averages, and those of the afternoon hours for the P.M. ones. The following results deserve attention:—

In the dog M (weighing, to start with, about 5 kg.), after food had been withheld for five days and insulin for the last two of them, the R.Q. gradually fell from 0·863 to 0·673, and the average energy expenditure from 76·6 per kg. to 73·2 per kg. At a later period in the same animal, when the body weight had been reduced to 3·6 kg., R.Q. fell to 0·674 in two days after insulin was discontinued, the average energy expenditure being 82 Cal. Later in this second series of observations (29th and 30th March), the animal was given meat and the quotient stood at 0·711, the D : N ratio being 4·72 on the first day, and 1.5 on the second. The ingestion of 40 gms. dextrose on the third day after insulin caused the R.Q. to increase from 0·662-0·735, but by the fifth day it had no effect, and on the sixth day it caused it to fall from 0·708-0·662. Similarly, during the second period of observation on this animal, 40 gms. dextrose caused the quotient to rise from 0·674-0·719 on the second day after insulin was discontinued, and had still a slight positive effect on the third.

TABLE

Date.	Condition.	24 hrs. Urine.			Respiratory Analysis.	
		D.	N.	D : N Ratio.	O_2 per Hr. and Kg. c.c.	R.Q.
Feb. 26	Last food, Feb. 25, 11 u. insulin .	—	—	—	655	0·863
,, 27	,, ,, ,, ,, .	—	1·85	—	640	0·757
,, 28	,, ,, ,, ,, .	—	1·77	—	—	—
Mch. 1	No insulin	—	2·07	—	—	—
,, 2	,, ...	—	2·44	—	647	0·673
,, 3	,, ...	8·08	1·6	—	912, A.M.	0·662
,, ,,	,, ...	—	—	—	759, P.M.	0·735
,, 4	,, ...	3·3	2·5	—	—	—
,, 5	,, ...	18·7	1·9	—	850, A.M.	0·661
,, ,,	,, ...	—	—	—	824, P.M.	0·666
,, 6	,, ...	20·8	2·1	—	709, A.M.	0·708
,, ,,	,, ...	—	—	—	823, P.M.	0·662
			Until March 24 given usual			
,, 26	No food or insulin since March 24 .	60	2·07	—	728, A.M.	0·674
,, ,,	,, ,, ,, ,, .	—	—	—	908, P.M.	0·719
,, 27	,, ,, ,, ,, .	47·8	1·35	—	803, A.M.	0·658
,, ,,	,, ,, ,, ,, .	—	—	—	974, P.M.	0·675
,, 28	,, ,, ,, ,, .	11·0	2·01	5·48	—	—
,, 29	250 gms. meat, but no insulin .	15·8	3·28	4·72	—	—
,, 30	450 ,, ,, ,, ,, .	12·0	7·95	1·5	908	0·711
Apl. 2	250 gms. meat + 40 gms. bactodextrose; no insulin . . .	44·8	4·8	—	822	0·72

TABLE

Mch. 17	Last food and insulin . . .	—	—	—	—	—
,, 19	No food or insulin since March 17.	7·75	2·88	2·79	591	0·737
,, 20	,, ,, ,,	—	—	—	614, A.M.	0·667
,, ,,	,, ,, ,,	—	—	—	684, P.M.	0·663
,, 22	,, ,, ,,	6·83	3·48	1·91	603, A.M.	0·644
,, ,,	,, ,, ,,	—	—	—	634, P.M.	0·648
,, 23	,, ,, ,,	53·7	4·31	—	609	0·640
,, 24	,, ,, ,,	4·55	3·0	1·52	—	—
,, 25	,, ,, ,,	27·0	—	—	670, A.M.	—
,, ,,	,, ,, ,,	—	—	—	689, P.M.	0·636

During both periods there was a steady increase in the average calorie expenditure, accompanied by a decline in body weight. It is, of course, difficult, in the dog, to rule out the effect of muscular exercise, and this, along with the decline in body weight, may account for the rise in energy expenditure. There is no evidence of any specific dynamic action when the dextrose was given, although this is evident after protein, as is seen by comparison of the results of 26th and 27th March (A.M.) and 30th March (all day).

In the second animal (L) very similar results were obtained.

XI.

Average Cal. per Kg. and Hr.	Body Weight Kg.	Movements.	Remarks.
76·6	5·216	Quiet.	M. ———
73·7	4·910	,,	———
—	—	——	Convulsions.
—	—	——	———
73·2	4·250	Quiet.	———
103	4·250	Decidedly restless	———
85·9	—	Quiet.	39 gms. bactodextrose at noon.
—	—	——	———
95·6	4·010	Decidedly restless.	———
92·7	—	Quiet then restless.	40 gms. bactodextrose at noon.
79·8	3·950	Occasionally restless.	———
92·6	—	,, ,,	40 gms. bactodextrose at noon.
quantities	of food a	nd insulin daily.	
81·9	3·63	Mainly quiet.	———
102	—	Very restless.	40 gms. bactodextrose at noon.
90·3	3·345	Some restlessness.	———
110	—	Restless.	40 gms. bactodextrose at noon.
—	—	——	———
—	—	——	———
102·2	3·175	Quiet all day.	(40 gms. bactodextrose equals 36·5 gms. glucose.)
92·8	2·860	Quiet but vomited.	———

XIA.

—	—	——	L. ———
67	5·046	Quiet except first hour	———
69·1	—	Mainly quiet.	———
76·9	4·990	——	40 gms. bactodextrose at noon.
67·8	—	——	———
71·3	4·420	Quiet.	40 gms. bactodextrose at noon.
68·5	4·310	,,	———
—	—	——	———
75·4	—	——	———
77·5	3·930	——	———

From the third day on after insulin, R.Q. fell gradually from 0·667-0·636, and was not influenced by giving dextrose, the energy expenditure rose slightly, probably in proportion to the decline in body weight. The D : N ratio varied between 2·79 and 1·52.

There is no evidence in these results of sugar formation from fat during starvation or exclusive meat feeding; under these conditions R.Q.'s lower than 0·66 were not observed. On the other hand, quotients varying from 0·648-0·636 were consistently obtained in the dog L. during four days when she was

receiving glucose, and although the D : N ratios, after making the necessary allowance for the ingested sugar, are far below those required to demonstrate gluconeogenesis from fat, the result indicates that this type of observation is worthy of further repetition. Observations on the quotient in dogs made diabetic by phlorhizin will be found in Lusk's "Science of Nutrition." Quotients lower than 0·66 have not been recorded.

The Respiratory Quotient in Hibernating Animals.—It has generally been considered that there is at least one clear case in which the R.Q. indicates gluconeogenesis from fat, namely, during the winter sleep of hibernating animals. It is said to fall to a very low level, while during this time large quantities of fat, which had been deposited in the tissues during the fall months, are becoming gradually used up without any change occurring in the percentage of glycogen. If we assume, as we must, that carbohydrates are essential for life, they must be derived from the protein or the fat of the animal's body. That protein cannot be the source is illustrated by the extremely low excretion of nitrogen, which occurs during the sleep. Indeed, in the marmot this is stated to be reduced to 0·025 gms. per kilogram, which is about ten times smaller than that of the starving animal after awakening. Based on a comparison of the nitrogen and CO_2, Nagai (1909) calculated that about 10 per cent. of the total energy comes from protein and 90 per cent. from fat. Although Nagai denies that the glycogen found in the liver is derived from fat, yet it seems clear from his own figures that this must be the case. Other observers, such as Voit and Pembrey (1901), have observed that the body weight of the animal may actually increase for short periods of time during the sleep, although usually it declines at the rate of about 2 gms. per day. No other explanation seems possible than that some of the fat has become partially oxidised to form sugar, which then becomes condensed to glycogen.

Although these facts would seem to demonstrate that carbohydrates may be formed from fat in hibernating animals, it should be pointed out that some observers, such, for example, as Hari (1909), call the accuracy of the published results in question. This author explains the low R.Q. as being due to the retention of the respiratory gases in the blood as the result of low temperature, infrequent breathing, etc. Magnus Levy

also doubts the accuracy of the low quotients, mainly for technical reasons; and Rubner (1924), in a recent critical survey of the researches on respiratory exchange, has shown, when the results of doubtful accuracy are eliminated, that there remains no evidence to show that the metabolism of animals during hibernation is in any way different from that of cold-blooded animals under similar temperature conditions. An excellent review of this and related work will be found in the monograph by Morgulis.

During the past winter we observed the respiratory exchange of a ground hog (*Arctomys monax*), the nearest American relative of the marmot of Europe. In the first experiments the same respiratory cabinet was employed as for dogs (p. 124), the space between the walls being packed with ice, and the whole apparatus kept in a room with the temperature near the freezing-point. Under these conditions the animal remained for periods of a week or two in profound sleep, breathing, as a rule, about twice in three minutes, and with an extremely low respiratory exchange. Indeed, this was so low that it was extremely difficult to make certain of the reliability of the results which were obtained. During several periods of six to eight hours, quotients varying between 0·64 and 0·66 were observed, but during others they were much lower, which might be accounted for by a slight error in the measurement of the oxygen consumption. On account of these difficulties, further use of the respiratory cabinet was abandoned, and in its place a Tissot-Carpenter spirometer of 100 litres capacity was tried. The animal could remain asleep in the spirometer for two or three days without any danger of being affected by accumulation of CO_2 or deficiency of oxygen, and the R.Q. was obtained by analysis in a Haldane apparatus of a well-mixed sample of the confined air. By this method quotients varying between 0·600 and 0·638 were obtained, but their accuracy was still questioned, because of inequality in the rates of diffusion of O_2 and CO_2 through the water seal and the outside air. Finally, an anatomical museum jar of 72 litres capacity, with a mercury seal and with a contrivance for thoroughly mixing the confined air was used, but only a few analyses were possible before the animal awoke from its sleep. The results finally obtained in this last experiment were as follows :—

Expt. *a*. After 26 hours in chamber at 5° C. breathing was 1 per min., O_2 – 19·22 per cent., R.Q. 0·71.

Expt. *b*. After 7 hours in chamber at 8° C. breathing was 2 per min., O_2 – 18·61 per cent., R.Q. 0·672 per cent.

Neither in the starving, depancreatised animal nor in hibernating animals have we so far been able to observe respiratory quotients which would unquestionably prove that carbohydrate has been formed from fat. On the other hand, when the conditions laid down by Geelmuyden were fulfilled—namely, that preformed carbohydrate should be fed to the animal during the time that the respiratory observations are made—quotients decidedly lower than 0·66 were obtained, in Dog L., on four consecutive days. The next obvious step is to determine the influence of insulin in experiments of this type.

In the hibernating animal, as Geelmuyden has pointed out, it is unlikely that satisfactory evidence of gluconeogenesis from fat will be obtained, since it is impossible to know with certainty whether the glycogen remaining in the body after the sleep has been produced during it, or represents what is left of the stores originally present. It is therefore extremely doubtful whether the results of the respiratory exchange of hibernating animals can be accepted as evidence of the conversion of fat into carbohydrate in the animal body.

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CHAPTER XI.

GLYCOGEN.

In 1848 Claude Bernard discovered that an extract made from the liver immediately after death contained much less sugar than one made several hours later, and he concluded that the organ must contain some reserve form of carbohydrate. He observed, further, that the agent responsible for this production of sugar must be of the nature of a ferment, since boiling the liver immediately after death prevented the process. He subsequently found, by extracting the organ with weak alkali and adding alcohol to the extract, that a substance was precipitated from which, by hydrolysis, sugar was obtained. This substance he named glycogen.

It is impossible to over-estimate the importance of this discovery, for, as Claude Bernard supposed, the formation of glycogen is the first step in the metabolism of the carbohydrates, and the glycogen also serves as an important reserve form of this proximate principle of food in the body. One may say, indeed, that carbohydrate in its metabolism in the animal body starts at the glycogen stage and ends up in the products of complete combustion. Between glycogen and the glucose derivatives which undergo ultimate oxidation, there are, no doubt, numerous intermediary substances, the identification of which has, however, not been clearly established. The chief reason for this difficulty is probably that the katabolic process is a dynamic and not a static one; a process of constant change, so that, with the exception of lactic acid, none of the intermediary substances accumulate in sufficient amounts in the body so that their presence can be detected by chemical means.

Chemical Properties of Glycogen.—It must be remembered that glycogen may not be a definite chemical substance, but rather a mixture of substances, characterised from several other polysaccharides only by its solubilities and a few colour tests.

Its separation from the tissues in which it is contained depends on heating these for several hours in the presence of 30 per cent. caustic potash, then diluting the solution and precipitating the glycogen with several volumes of alcohol. The precipitate is re-dissolved in water, re-precipitated with alcohol, the final watery solution hydrolysed by heating with 2 per cent. hydrochloric acid and the glycogen determined as dextrose. To obtain comparable results, it is necessary that the chemical procedures be carried out exactly alike in all the estimations. The final precipitate obtained by alcohol can be dried *in vacuo* over phosphorus pentoxide. Being a colloid, glycogen can be largely freed from inorganic salts by dialysis, when its precipitability by means of alcohol becomes much less marked, unless concentrated solutions are used. A dilute solution has a specific rotatory power of 196·57°, and the heat of combustion is 3883 Cal. per gramme of the dried alcohol precipitate (Slater, 1923). Glycogen is very closely related to the higher dextrines and, like them, it gives a brownish-red colour with iodine, but is distinguished by the fact that it forms strongly opalescent solutions, instead of clear ones. The lower dextrines are not likely to be present in preparations of glycogen, partly because they are not so readily precipitable, and partly because, like sugars, they are destroyed by caustic alkali.

Glycogen can be identified in sections of tissues which have been fixed in alcohol, either by treating with iodine solutions containing gum acàcia (this being added to prevent solution of the glycogen), or by staining with carmine, by Best's method. This latter method has proved to be one of great value for micro-chemical purposes, and is carried out by first overstaining the tissues with carmine and then partially decolorising by means of sodium sulphite. The glycogen retains the red stain under these conditions and, when present in any considerable amount, appears in the cells as small, often irregularly outlined clumps of material which can be dissolved, either by washing the tissue with water or by treating with saliva. The amount of glycogen in the tissues as determined by chemical means compares fairly well with that estimated by histological methods, thus contrasting with fat, for which the amounts found by chemical means may be entirely different from those estimated as a result of histological observation.

Distribution in the Tissues of Higher Animals.—The highest concentration of glycogen, under average conditions of nutrition, occurs in the liver. When small amounts are present it occurs mainly in the cells situated towards the centre of the lobule, although a narrow fringe of cells at the periphery may also contain it. When the amount is large the cells throughout the lobule may be filled with glycogen. It is never present in the nuclei. Under certain conditions material staining like glycogen, with Best's carmine method, may also be seen in the intercellular

capillaries, in the Kuppfer cells, and sometimes in the larger venules. Thus, Ishimori (1913) found it in these positions after rich feeding, and Huber and Macleod (1917), after inducing a rapid breakdown of glycogen, and consequently hyperglycæmia, by piqûre in rabbits. Under ordinary conditions of feeding, the liver of the rabbit commonly contains from 5-7 per cent. of glycogen, and in the dog about 3 or 4 per cent. By rich carbohydrate feeding as much as 12 per cent. may be present. Assuming the liver to be about 4-5 per cent. of the body weight in the rabbit, not much more than a total of 8-10 gms. of glycogen can be present in the liver of an animal of average weight. The liver of man can rarely contain more than 5 per cent. of glycogen, giving a total of about 150 gms. Since this is a small amount compared with the total of carbohydrate assimilated under ordinary dietetic conditions, it is clear that a great proportion of the carbohydrate storage in the body must occur elsewhere than in the liver.

The distribution of glycogen in different portions of the liver is almost exactly uniform, at least in the rabbit and dog; it may, however, be unequal in the liver of cold-blooded animals, such as the turtle.

This is illustrated in the following table, in which typical results obtained on two animals of each class are given :—

Animal.	Portion of Liver.	Per Cent. Glycogen.	
		I.	II.
Rabbit	Quadrate lobe	11·05 ;	8·84
	Left lobe	10·80 ;	8·79
Dog	Left lobe	10·845 ;	14·445
	Left central	10·890 ;	14·135
	Caudate	10·710 ;	14·840
	Right central	11·350 ;	14·700
	Right lobe	11·610 ;	14·065
Turtle	Right lobe	6·63 ;	7·59
	Left lobe	5·66 ;	6·77

(*From* MACLEOD and NOBLE and MACLEOD and PEARCE.)

Since breakdown of glycogen occurs very rapidly after death it is necessary to remove the portions of liver from each lobe as nearly at the same time as possible to obtain these results.

The muscles are the other main seat of carbohydrate storage, but the precentage here is considerably less than in the liver, very seldom being above one. Following starvation, glycogen still

remains in the muscles after it has practically disappeared from the liver. There is, however, great variability in the percentage of glycogen in different muscles, as will be evident from the following table from Cremer :—

Dog. I.—*Biceps brachii* . . .	0·17
Quadriceps femoris . .	0·53
Dog. II.—*Biceps brachii* . . .	0·25
Quadriceps femoris . .	0·32

This inequality in distribution of glycogen makes it impossible to compute the amount in the entire musculature from analysis of a few muscles. Its cause is related to the utilisation of this material during muscular contraction. By micro-chemical methods the glycogen in muscle is seen to be present in the fibres, both in the sarco substance and the sarcoplasm, unless only small amounts exist, when it is restricted to the sarcoplasm.

The distribution of glycogen, in the other tissues of the mammal, is indicated in the following table, which gives the results obtained by Schöndorff in seven dogs that had been well fed with carbohydrate shortly before death :—

	Maximum Observed.	Minimum Observed.	Average.
Blood . . .	0·04	0·001	0·015
Liver . . .	56·74	20·09	37·97
Muscle . . .	62·55	31·22	44·23
Bone . . .	12·88	5·36	9·25
Skin . . .	11·38	1·42	4·49
Viscera . . .	7·30	0·38	3·81
Heart . . .	0·28	0·08	0·17
Brain . . .	0·23	0·04	0·09
Total . . .			100·0

Distribution in the Lower Animals.—Glycogen occurs in the tissues of all animals, with the possible exception of certain of the mollusca. (For comprehensive reviews, see Pflüger and Biedermann.)

The earlier work depended on the use mainly of histological methods, but more recently chemical methods have been employed. It has been isolated in the protozoa, particularly in cultures of *Glaucoma scintillans*. Peculiar transparent substances, called "Glanzkörper," present particularly in pelomyxa, are also believed to consist of glycogen, partly because they give an iodine reaction and partly because they become increased when the animal is fed with polysaccharides. Typical glycogen has been described in certain Echinodermata (asteroids, Holothurians), and in sponges, and it has been identified by micro-chemical methods in the eggs of insects and molluscs. It has long been

known that yeast contains glycogen (cf. Cremer, 1902), and Harden and Young (1902) have isolated from washed, pressed yeast, by the usual method, as much as 2 per cent. of glycogen which, in all its chemical characteristics, was identical with that prepared from rabbit liver and from oysters.

There can be no doubt, then, that in the lowest forms of animal life a material corresponding to, if not identical with, the glycogen of higher animals is present in considerable amounts. In some of the lower invertebrates the amounts are often very large. Weinland, for example, found that about one-third of the weight of dried Ascaris consisted of glycogen. In the digestive gland of various mollusca considerable quantities are well known to be present. In Table XII. is shown the glycogen percentage found in various of the lower animals.

It is of some interest that several observers (Frentzel, Röhmann, Bottazzi) have been unable, either by micro-chemical or bio-chemical methods, to detect any glycogen in the bivalve Aplysia (the sea-hare). This mollusc lives mainly on an Alga (*Ulva lactuca*), which contains large quantities of pentosan, and it has been suggested that carbohydrate is stored in these animals as an acid form of pentosan (*acide pentosique*), instead of as glycogen. In Octopods also it is said to be impossible to find glycogen. It is probable that a technical error is responsible for these negative results. Henze and Starkenstein attribute this to the formation of magnesium hydroxide which adsorbs the glycogen so that it is not dissolved by boiling water. By taking precautions to avoid this source of error these workers found considerable quantities of glycogen in the liver of Aplysia. In various marine invertebrates and fishes Kilborn and Macleod (1920) obtained the results which are shown in Table XII. The variability in the amounts found, particularly in the fishes, is, no doubt, dependent, partly on the feeding conditions and partly on muscular activity. Thus Schöndorff and Wachholder (1914), working on fresh-water fishes, observed that prolonged hunger causes considerable reduction in the amount of glycogen in those which remain active, such as the pike (*Esox lucius*), but only slight reduction in those which hide themselves away in the mud during winter, such as the carp (*Cyprinus carpio*).

The relatively large amount of glycogen in the primitive heart, particularly of the dog-fish—in which the percentage is often greater than in the liver—is of interest in view of the fact that similar, relatively large quantities of glycogen have been shown, by histo-chemical methods, to exist in the conducting tissues (A-V node and A-V bundle) of the mammalian heart.

TABLE XII.

Group.	Species.	Organ or Tissue.	Wt. of Material. Gm.	Glycogen Percentages.	
				Calculated for Original Material.	Calculated for Alcohol Preserved Material.
Echinodermata (Asteroidea)	Pisaster ochrasea	Hepatic cæca	41·9	1·23	1·52
,,	Pisaster brevispinus	,,	6·0	—	0·232
,,	Luidia foliata	,,	22·0	—	0·461
Mollusca (Lamellibranchiata)	—	Digestive gland	22·5	—	1·56
,,	—	Muscle of siphon	30·0	—	0·952
,,	—	Muscle of foot	19·4	—	1·70
,,	—	Adductor muscles	20·6	—	2·67 (?)
Arthropoda (Crustacea)	Cancer productus	Liver	—	—	1·39
,,	,,	Muscles	24·65	—	0·87
,,	Homarus	Liver	35·64	0·78	—
,,	,,	I. Heart	2·37	0·91	—
,,	,,	II. ,,	1·94	1·42	—
,,	,,	I. Muscle (tail)	55·0	0·36	—
,,	,,	,, (claw)	50·0	0·17	—
,,	,,	Heart (of six lobsters)	7·32	0·85	—
,,	,,	Liver	30·0	0·05	—
,,	,,	Muscle (back)	20·0	0·31	—
The Fishes (Elasmobranchii and Teleostomi)	Elasmo. (Squalus sucklii) dog-fish	Liver	44·2	0·057	0·069
,,	,,	,,	20·0	0·16	0·209
,,	,,	Muscle	19·8	None	None
,,	,,	Heart	7·3	0·447	0·847
,,	Chimæra rat-fish	Liver	16·7	None	—
,,	Teleostomi (Cyprinus carpio), carp	Liver	3·44	Trace	—
,,	,,	Muscle	50·0	,,	—
,,	,,	Liver	10·0	6·50	—
,,	,,	Muscle	20·0	0·29	—
,,	,,	Liver	10·0	5·60	—
,,	,,	Muscle	20·0	Trace	—
,,	Christivomer Namaycush (lake trout)	Liver	26·6	None	—
,,	,,	Muscle	13·91	Trace	—

(KILBORN and MACLEOD.)

As is well known, this conductive tissue is the homologue of the primitive heart of the dog-fish.

The great variability observed in the amount of glycogen present in the livers of fishes of different species raises the question as to whether the amount is the same in those of different individuals of the same species. If the variability, as suggested above, depends on feeding and muscular activity, then it should be much less pronounced when specimens of the same species caught under the same conditions are compared. McCormick and Macleod (1925) have, however, obtained data which show that the same variability exists (*Myoxocephalus*). These fish were caught at the same feeding grounds and at the same period of the year (August and September), and the following results were obtained :—

TABLE XIII.

Date.	Wt. of Fish. Gm.	Per Cent. Liver Wt. to Body Wt.	Glycogen in Liver. Per Cent.
10 Sep.	208	3·8	12·0
,,	177	1·5	6·9
,,	396	5·3	8·7
26 Aug.	269	2·9	5·6
29 ,,	312	5·0	0·11
,,	340	2·4	3·76

Glycogen in Plants.—Since it is possible that much may be learned of the physiological significance of glycogen by observing its behaviour in the simplest forms of cells, some attention has been paid to its presence under various conditions in certain chlorophyll-free plants. In the yeast cell, glycogen exists as either granules or droplets diffusely scattered through the protoplasm. Certain yeasts, however, are said never to form glycogen, such as *Saccharomycosis exiguus* (Lindner and Henneberg, cf. Biedermann).

Formation of glycogen has been carefully investigated in relationship to the composition of the nutritive medium in which the yeast is grown, and it has been found that it occurs in the presence of lactic, succinic, and tartaric acids, the amino bodies, asparagin, and glutamine. It is not formed in the presence of glycerol, lactose, or the pentoses (Laurent, 1890). The glycogen in yeast has usually been considered as a reserve carbohydrate, and in this connection it is interesting to

note that Pasteur attributed to the stored carbohydrate in yeast cells, the alcohol and CO_2 which was formed during alcoholic fermentation in excess of that which could be accounted for by the sugar present in the solution and he succeeded in extracting fermentable sugar from yeast with weak sulphuric acid. It is to this glycogen, also, that the fermentation of alcohol, which occurs in washed yeast by incubating it at fairly high temperatures, is to be attributed. Although the intracellular glycogen in yeast is readily hydrolysed, glycogen added to the fluid in which yeast cells are growing is not affected. But the glycogen in yeast may not, as is usually assumed, be merely a reserve material, but also an important intermediary product in the process of alcoholic fermentation, as has been suggested by H. G. Kohl (1908). This worker believes that the hexoses are split up into alcohol and carbon dioxide only after a glycogen stage has been gone through. If this should prove to be the case, it would lend support to the view which is gaining ground that glycogen plays a similar rôle in the metabolism of animals (see p. 179).

Besides being present in yeast, glycogen also occurs in various other plants. It can be prepared in considerable quantities from such fungi as *Boletus edulis*, *Amanita muscaria*, and *Phollus impudicus*. In certain of these fungi it undergoes rapid hydrolysis as a result of the action of enzymes.

At an early stage in the investigations of the action of insulin, it appeared as if useful information might be obtained by ascertaining the influence of this hormone on the growth and activity of yeast. Noble and I added it in varying proportions with well buffered suspensions of yeast and sugar, without finding the slightest indication that it had any effect. This has since been confirmed by Travell and Behre (1924), by von Fürth (1924), and by Laufberger (1924). It is improbable, therefore, that its addition to a yeast culture could influence glycogen formation.

The Formation of Glycogen in the Higher Animals.—This is investigated by first of all rendering the animal as free of glycogen as possible, then feeding it with the substance whose influence on glycogen formation is being investigated, and measuring the amount of glycogen present, either in the entire animal or in the chief organs in which it is deposited, the liver and muscles. In the former case, only small animals, such as mice, can be used, and in the latter, it is usual to choose the organ which is richest in glycogen, namely, the liver. This is called the *direct* method in contrast to the *indirect* method, which is based on the fact, readily demonstrable by the direct method,

that glucose forms glycogen. It consists, therefore, in determining whether or not the substance in question is capable of forming glucose when it is given to a diabetic animal.

Simple though the direct method may appear to be in theory, it has proved itself to be one of considerable uncertainty. The first step consists in removing as much glycogen as possible from the animal, and it is here that much of the uncertainty creeps in. Starvation alone cannot be depended on, for although the liver usually becomes practically free of glycogen after about four days in the rabbit or dog, this is not invariably the case, and if the starvation be prolonged beyond four days it is not unusual for higher percentages of glycogen to be found than were present at earlier stages of starvation. On account of the varying conditions of these animals prior to the starvation, particularly with regard to feeding, confinement, exercise, etc., and also because they are not uniform in age, sex, or breed, this irregularity in glycogen content is not to be wondered at. In order to eliminate these sources of irregularity as far as possible, Külz used hens and found, after six days' starvation, the following amounts of glycogen in the entire animal: 0·701, 0·543, 0·042, 0·335, 1·394, 1·079, and 1·761 grms. Using rabbits, as nearly as possible alike in breed and size, McCormick and I (1923) found in the liver, heart, and muscles, after two to four days' starvation, the following percentages :—

Duration of Starvation.	Percentage of Glycogen in:		
	Heart.	Muscles.	Liver.
3 days (plus epinephrin)	0·62	0·05	0·30
,, ,,	—	0·01	0·26
,, ,,	0·13	0·05	0·08
,, ,,	0·18	trace	0·52
4 days (no epinephrin)	0·52	0·06-0·10	0·01

On account of these unsatisfactory results, Karczag, Macleod, and Orr (1925) have used the standard white rat, kept under the strict conditions of feeding and caging recommended by the Wistar Institute for Anatomy. Since different individuals are of constant anatomical build, and their blood sugars almost exactly the same, it was to be expected that the glycogen, after a suitable period of starvation (twenty-four hours) would also be alike. The following results were obtained :—

Wt. of Rat. Gm.	Per Cent. Blood Sugar.	Per Cent. Glycogen in: Liver.	Muscles.	
267	0·102	0·48	0·28	Males, killed on same day
230	0·102	0·26	0·35	
232	0·102	0·21	0·24	
275	0·106	0·20	0·26	
270	0·096	0·17	0·33	
270	0·106	0·18	0·29	
190	0·103	0·12	0·28	Females, killed on same day
175	0·106	0·13	0·29	
196	0·106	0·11	0·26	
187	0·103	0·10	0·22	
180	0·106	0·10	0·28	
183	—	0·23	0·28	

These results are satisfactory. They show that in the standard white rat, starved for twenty-four hours, a suitable test object is obtained, by the use of which the influence of various conditions on the formation of glycogen may be studied. It is almost certain that the glycogen, which disappears from the liver during the earlier stages of starvation, is replaced by more which appears later, being produced, no doubt, as a result of the processes of gluconeogenesis which are known to occur.

If the starvation be combined with muscular exercise, removal of glycogen from the liver becomes much more complete. In the case of large animals (dogs), work on a treadmill is a satisfactory method, but for smaller ones (rabbits) the muscular contractions are most conveniently induced by means of strychnine in sufficient dosage to cause convulsive contractions of the muscles of the extremities, without tetanus of those of respiration. If this should threaten, artificial respiration must be applied until the danger of asphyxia is over. After the convulsions have lasted for two to three hours, in a previously starved rabbit, the liver can be depended upon to be glycogen-free, provided the animal is immediately killed, but if the convulsions be antidoted by injecting chloral hydrate (per rectum), glycogen is quickly formed again, so that in twenty-four hours 2 or 3 per cent. may accumulate in the liver. The muscular action, in either large or small animals, may also be brought about by causing the rate of heat loss from the surface of the body to become increased, the usual method employed for this purpose being to give the animal a cold bath, and then keep it for some hours in a cool room.

Finally, the glycogen can be cleared out of the liver with certainty in starved animals by giving them phlorhizin, which acts by draining the sugar out of the body so that the glycogen stores are called upon to replace the lost glucose. We have found this a very satisfactory method, much more so than that depending on administration of epinephrin, which does not remove the glycogen with certainty. Indeed, as has been shown by Pollak (1909) and by Markowitz (1925), that when epinephrin is administered to *a*-glycogenic animals, it may cause glycogen to reappear in the liver. The significance of this observation will be discussed later (p. 228).

Quite apart from these difficulties in making certain that the liver is free of glycogen to start with, the direct method can never be a very dependable one, since it merely tells us how much glycogen is *left* in the liver, and not how much has been *formed* during the process of assimilating the food. It is conceivable, for example, that any glycogen which may be formed in the liver is so quickly used up that none of it is deposited.

With regard to results, it can readily be shown that feeding with glucose, or with any sugar which during its digestion leads to the absorption of glucose, causes glycogen to be deposited in large amounts in the liver, and the question arises as to whether this occurs uniformly in all the lobes or more quickly in certain of them than in others?

Some years ago Sérégé (1905) stated that the right lobes of the liver fill up with glycogen more quickly than the left, and he explained this as being due to the fact that the blood streams of the splenic and mesenteric veins do not become completely mixed together in the portal vein, so that, at the hilus of the liver, a greater proportion of the splenic vein blood goes into the left lobes and a greater proportion of mesenteric vein blood into the right. Although this incomplete intermixing of these bloods can be demonstrated under certain conditions, there is no evidence to show that it results in any inequality of glycogen deposition, as Sérégé supposed. His own observations are open to question, on account of the inaccuracy of the method which he employed for the estimation of glycogen. They have been repeated by Pearce and Macleod (1913), with results which show that the deposition is tolerably uniform. The experiments consisted in feeding, to previously starved dogs, definite quantities of cane sugar and then determining the amounts of glycogen in the various lobes of the liver at regular intervals, precautions being taken to obviate errors due to postmortem glycogenolysis. In two animals killed in six and twelve hours

respectively after feeding, the following results were obtained in the different lobes :—

TABLE XIV.

THE DISTRIBUTION OF GLYCOGEN IN THE LIVER.

Dog.	Time after Feeding at which Dog was Killed.	Per Cent. of Glycogen in Lobes.						Greatest Difference in per cent. of Largest Amt.
		Left.	Left Central.	Caudate.	Right Central.	Right.	Greatest Difference.	
1	1 hr. .	3·515	3·549	3·485	3·335	3·045	0·549	15·5
2	3 hrs. .	4·635	4·171	4·104	4·149	4·126	0·531	11·2
3	5 hrs. (and 1 hr.) *	3·481	3·468	3·312	3·094	3·054	0·427	12·2
4	7 hrs. (and 3 hrs.) .	9·393	10·026	10·198	9·721	8·970	1·228	12·04
5	18 hrs. .	7·525	6·708	6·725	6·801	6·700	0·825	10·9

* The times given in brackets indicate when a second administration of food was given.

It is possible that at earlier stages in the process of absorption a certain degree of unequal filling of the lobes may occur, but the differences cannot be significant.

Fructose also forms glycogen which is apparently of the same chemical composition as that formed from glucose, at least, it yields glucose and not fructose on hydrolysis. As judged from the relative amounts of glycogen found in the liver of diabetic animals, this sugar apparently forms glycogen more readily than glucose (p. 46). It is also significant that fructose is more easily metabolised by a partially diabetic animal than glucose. This is evidenced by the fact that it can cause the R.Q. to become raised under conditions where glucose has no effect in this regard (see p. 262).

Galactose, one of the monosaccharides of lactose, also forms glycogen, but apparently not so readily as glucose and fructose. Mannose probably also forms it, but I am not aware of experiments bearing particularly on this substance.

Glycogen Formation in the Isolated Perfused Liver.—An important question in metabolism is whether the formation of glycogen can occur in the liver or muscles independently of aid from other organs. Croftan (1909) stated, for example, that the glucose molecule must undergo some preliminary change before it can be converted by the liver cells into glycogen. He imagined that this change must occur, either during the absorption process

in the intestine, or by an action on the sugar after it gained entry to the blood. Winter and Smith, also, have more recently claimed that a mixture of liver extract and insulin, but not liver extract alone, is capable of converting an equilibrated mixture of α-, β-glucose into a more reactive, unstable form—γ-glucose—which they assume would then readily condense to form glycogen. It is clear that these suggestions can be put to the crucial test most satisfactorily by seeing whether glycogen is formed during perfusion of the isolated viscus.

Perfusion of the Liver of the Turtle.—Grube called attention to the possibility of using the liver of the turtle for the study of glycogen formation, because of the fact that its main blood supply is carried to the two lobes by separate branches of the umbilical vein. The two lobes are connected together by a narrow isthmus of liver tissue, so that by tying a ligature around this, one lobe can be removed and the glycogen determined in it, and the other perfused with Ringer's solution containing the substance whose influence on glycogen formation it is desired to investigate.

Because of the extensive glycogenolysis occurring in the perfused lobe, however, such comparisons are unreliable, the glycogenolysis usually outweighing the glycogenesis. The method has, therefore, been modified so that each lobe is perfused separately, the substance in question being added to the fluid on one side only. In this modification it is assumed that glycogenolysis will proceed at an equal rate in both lobes, so that, if the added substance should have a glycogenetic effect, a difference will be found in the amounts of glycogen left in them after the perfusion. Even with this refinement, however, the method is not dependable because, as Schöndorff and Grebe (1911) have shown, there may be considerable differences in the glycogen content of the two lobes of the turtle liver, a fact which has been confirmed by Noble and Macleod (1923), who found the percentile differences in three cases to be 14·6, 10·8, and 3·4 respectively, the greater percentages being in the right lobe. Sometimes the difference may be much greater, Schondörff and Grebe finding it to be 32 per cent., for example.

The method is entirely unreliable for the detection of glycogen formation, unless possibly when this is very pronounced, as occurs when excess of glucose is present in the perfusion liquid.

Snyder, Martin, and Levin (1922) have employed the turtle liver to study the amount of glucose, or other sugar, which disappears from the perfusion fluid in fixed periods of time. When less sugar is found in the fluid after perfusion than that

originally present in it, evidence of retention by the liver is obtained, but it cannot be said whether this is as glycogen. On the other hand, if more sugar is found, it can be concluded that glycogenolysis has occurred.

In conducting the observations in this way, it is necessary that the small veins which run between the left lobe of the liver and the pancreas be mass-ligated, and that cannulæ be inserted into each of the umbilical veins and preferably also into the spermatic vein, which enters the right lobe towards the posterior edge of its lower aspect. If these precautions be not taken, the irrigation of the liver is very unequal—as can readily be seen by the irregular manner in which the blood is washed out of it—and considerable variations occur in the percentage of sugar in the outflowing fluid, because only sometimes does the irrigation fluid pass through the less accessible parts from which, however, it picks up large quantities of sugar, because of the glycogenolysis which has been going on there. Snyder, etc., found that a most important factor in controlling the rate of glycogenolysis is the rate of perfusion.

Perfusion of the Mammalian Liver.—Since glycogen formation cannot be satisfactorily investigated by using the turtle liver, except when marked glycogen-formers such as glucose and fructose are used, it is not to be expected that better results will be obtained with the mammalian liver. Even when every precaution is taken to conduct the perfusion so that physiological conditions are simulated, rapid glycogenolysis invariably occurs. Pearce and I (1911), for example, started the perfusion of the liver of the dog with fresh defibrinated blood, through a branch of the portal vein, before ligating the vein itself, peripheral to this branch. The liver was left *in situ* during the perfusion, its temperature carefully maintained at the normal level, and in several experiments, the hepatic artery was perfused with arterialised blood at a high pressure, while blood was simultaneously perfused through the portal vein at a low one. As a rule, the perfusion through the portal vein would proceed satisfactorily, at a pressure of about 10 mm. Hg., for about half an hour, and then a high degree of resistance would become developed, so that, to obtain any flow, the portal pressure had to be raised, with the result that marked œdema of the liver set in. Neither the addition of buffer substances (alkaline phosphates) nor the high arterialisation of the blood, had any beneficial influence on the perfusion. In no case was it found possible to demonstrate that glycogen had been formed in the

liver; on the contrary, post-mortem glycogenolysis set in as rapidly as in the excised liver placed in the incubator. It is, of course, possible that more satisfactory results might be obtained by using other animals than the dog.

J. de Meyer has attempted to demonstrate glycogen formation in the isolated mammalian liver by a somewhat different method. Recognising that it is impossible to prevent glycogenolysis by perfusing Locke's solution, he attempted to compare the rate at which this proceeds in different lobes by perfusing them separately, one with Locke's solution alone, and another with this *plus* the substance, whose influence on glycogen formation it was desired to study. It was argued that a comparison of the glycogen content in the different lobes would reveal whether glycogen formation had taken place, for, although glycogenolysis would occur in both, it would be lessened in that lobe in which glycogen formation was also occurring. Meyer's results do not, however, fulfil these expectations for, as might be expected, post-mortem glycogenolysis is so prepotent a process that nothing can stay it. Pavy and Siau had previously shown that it could be delayed by the injection of alkaline solutions into the portal circulation, but apart from this it is certain that no one has yet succeeded in showing that the process can be perceptibly delayed by any procedure which can be considered as in any sense physiological in nature.

It has so far been impossible to demonstrate that glycogen formation occurs during perfusion of the liver with fluid containing no hormone, or other agency derived from other organs. Some is probably formed in the turtle liver when the perfusion fluid contains a considerable concentration of glucose or fructose, as Grube's results would seem to show, but it is possible that there still remained in the liver cells sufficient quantities of some hormone, derived during life from other organs, to polymerise these sugars. Such hormones would probably not remain for any length of time in the case of the mammalian liver.

Glycogenolysis.—In the chain of chemical processes which take place between the absorption of glucose from the gastro-intestinal tract and its final oxidation in the tissues, the breakdown of glycogen into glucose is one of great importance. The rate of this glycogenolytic process, as it is called, is so regulated that glucose is supplied to the systemic blood in proportion as its concentration tends to fall, and one of the most important problems of carbohydrate metabolism is with regard to the nature of the controlling mechanism by which this is brought about. Is the rate of glycogenolysis regulated merely by changes in the

composition of the blood which bathes the liver cells, or is it dependent upon nervous impulses carried to the organ by the autonomic nervous system? If changes in the blood should be responsible, are these dependent on the concentration of glucose itself, or on the presence of other substances derived from glucose during its breakdown? The significance of those questions in connection with the effect of insulin on the level of blood sugar is obvious, and will be discussed in the next chapter.

In all forms of hyperglycæmia that are not due to excessive absorption of glucose from the alimentary canal, the source of the glucose is at first the glycogen of the liver. In those which are produced in laboratory animals by stimulation of the nerve supply of the liver, or by asphyxial conditions, the hyperglycæmia usually ceases when all of the glycogen of the liver has been used up, thus contrasting sharply with the hyperglycæmia which follows pancreatectomy, in which a process of gluconeogenesis supervenes upon that of glycogenolysis. In epinephrin hyperglycæmia, however, sugar continues to be formed after the glycogen has disappeared.

The agent responsible for the hydrolysis of glycogen is, of course, a diastatic enzyme, which we may call *glycogenase*, and our first problem must be concerned with the laws which govern its action and the conditions which influence them. We shall then consider its distribution in the various organs of the body, its source (i.e. whether produced locally wherever it is found present, or whether derived from some one organ and distributed to the others), and its quantitative behaviour in the body in those various conditions in which glycogenolysis occurs.

Methods of Studying the Action of Glycogenase.—The action of glycogenase, like that of other enzymes, may be measured, either by determination from time to time, of the amount of glycogen left unchanged (substrat) or of the amount of sugar formed.

The latter method is more or less inaccurate because, in the breakdown process (hydrolysis) here involved, many intermediary products (dextrines) are formed, some of which have a reducing action. The final product of the hydrolysis, maltose, is also liable to be hydrolysed further to glucose, which, besides having a different reducing power from maltose, is liable to be attacked by glycolytic enzymes. In such a complex mixture of reducing substances it is not to be expected that the rate at which the original glycogen is becoming hydrolysed could be

as accurately determined as by measurement of the amount of substrat remaining in the incubated mixture at regular intervals.

The measurement of glycogen being somewhat laborious, several workers have used as the substrat, not glycogen, but starch, because the hydrolysis in this case can be readily followed by employing the sensitive starch-blue reaction with iodine, and Salkowski and Wohlgemuth have elaborated satisfactory procedures for using this method quantitatively. In using it, however, it is assumed that glycogenase acts exactly like amylase, and it is by no means certain that this is the case. No doubt diastases which can act on amylose will also be able to act on glycogen, but there may be varieties which are feebler and can only attack glycogen and the dextrines. This possibility is sustained by the well-known fact that specific enzymes exist for each of the common hexose disaccharides, cane sugar, lactose and maltose. Quite apart from these theoretical objections to using the starch-blue reaction, a practical difficulty arises when the glycogenolytic process is being followed in mixtures which contain extracts of various organs. The suspended particles of protein, etc., which are nearly always present in such mixtures absorb the iodine and make it difficult to determine the end point of the colour reaction.

In the present unsatisfactory state of our knowledge concerning the chemical structure of starch and glycogen, it is therefore much safer, in investigating the action of glycogenase, to determine the remaining glycogen by the Pflüger method. In doing this the difference between the amounts of glycogen present in the incubated mixture before and after incubation for varying periods, expressed as a percentage of the amount of glycogen initially present (percentile glycogenolysis) is ascertained.

Comparison of the Concentration of Glycogenase in Various Organs and Tissues.—This cannot be done with any degree of accuracy. When the concentration of glycogenase in the body fluids is under investigation, it is usual to employ measured quantities of them, but when it is desired to determine the concentration in solid tissues, or to compare it in blood and in tissue, it is necessary to adopt some arbitrary basis of comparison. For this purpose we have used the nitrogen content of the extracts or fluids.

With regard to the method of preparation of the extracts, that used by Buchner (hydraulic press) is satisfactory, but the extracts are very unstable, because they contain proteolytic enzymes which quickly destroy the glycogenase, even when the extracts are kept at a low temperature. For obtaining more permanent preparations, either the

alcohol process, or, better still, that of Wiechowski (1909), must be employed. In the former, some of the tissue is ground thoroughly in a mortar, using some quartz sand if necessary, and is then mixed with alcohol (several volumes). The resulting precipitate, after standing under the alcohol for a few days, is then collected on a filter paper and dried *in vacuo*. A weighed portion of the powder can then be taken at any time and ground up into a homogeneous suspension with water. The chief objection to the method is that contact with alcohol appears to alter the potency of the precipitate so that, if it is to be used for purposes of comparison, great care must be taken to see that all the conditions are exactly the same.

The Wiechowski method is the best in our experience. The tissue is very rapidly macerated to a fine pulp, which is then spread out on glass plates and these placed in a rapid current of warmed air until completely dry, when the crust is removed and the scales thoroughly extracted in a suitable extractor with toluol. This removes all traces of fat and the residue can be ground to a dust-fine powder, weighed portions of which, shaken with water, are used for the incubations. Minced tissue or simple watery extracts cannot be used.

The following table will serve to illustrate the relative efficiency of these methods :—

No.	Preparation.	Percentile Glycogenolysis in 2 hrs.	Percentile Glycogenolysis in 4 hrs.
1	Minced liver . .	16·3	—
2	Saline extract . .	9·7	12·3
3	Buchner extract . .	29·4	29·2
4	Air-dried liver powder .	29·2	45·9
5	Alcohol precipitate .	34·0	54·8

(MACLEOD.)

Influence of Reaction on the Action of Glycogenase.—There is probably no enzyme more sensitive to changes in reaction than diastase, the optimal reaction being slightly to the acid side of neutrality. When, therefore, the original reaction of a mixture of diastase and starch solution is neutral, or faintly alkaline, the addition of acid accelerates the hydrolysis, provided too much be not added, in which case retardation will occur; the addition of even a minute trace of alkali immediately depresses it. Addition of alkali to a mixture the reaction of which is acid will have an effect depending on the initial degree of acidity. If this be so high that it exceeds the optimum of acidity, alkali will cause acceleration, but if it be at or below this point retardation will occur. The great susceptibility of

diastases to the reaction of their environment is one of the chief causes for the very unsatisfactory nature of the results which have been published from time to time, with regard to the amount present in the animal tissues and fluids.

To illustrate the influence of reaction in connection with the glycogenase contained in liver extract and in blood serum, the following table is of interest :—

No. of Expt.	Degree of Acidity or Alkalinity.	Percentile Glycogenolysis in: Liver.	Serum.	Remarks.
T	Original reaction . .	63·8	42·8	1 c.c. serum or extract + 20 c.c. 1 per cent. glycogen solution incubated 3 hrs.
,,	0·0078 per cent. acetic acid	77·4	23·3	
,,	0·0224 ,, ,,	66·0	27·0	
,,	0·0390 ,, ,,	—	5·6	
A	Original reaction . .	26·9	29·4	Same as experiment T except that 2 c.c. liver extract used, and incubation continued for 4 hrs.
,,	0·2 c.c. 1 per cent. Na_2CO_3	19·6	28·1	
,,	0·4 c.c. 1 ,, ,,	6·6	23·6	
,,	0·6 c.c. 1 ,, ,,	9·6	24·9	
,,	1·0 c.c. 1 ,, ,,	—	9·0	

(MACLEOD.)

Although the actual pH of the mixtures could not be measured at the time when these observations were made, it is evident that extremely small quantities of acid have a considerable influence on the percentile glycogenolysis in both serum and liver.

These facts have their application in explaining the onset of glycogenolysis, which occurs in the liver immediately after death (post-mortem glycogenolysis), the acid being probably sarcolactic. When extracts of liver are employed in studies on glycogenase, the effects of the acid should therefore be prevented, by bringing considerable quantities of buffer solution in intimate contact with the liver cells, as soon after death as possible. The influence of hydrogen-ion concentration on glycogenolytic action is important also as a possible factor in explaining the excitation of this process which occurs during life. In various experimental conditions, such as asphyxia, vascular changes in the liver, etc., and in many other conditions, the possibility of intracellular changes in pH must always be borne in mind as a factor effecting the action of glycogenase.

Comparison of Strength of Glycogenase in Different Organs

of the Same Animal.—It is obvious, from what has just been said, that comparison of the concentration of glycogenase in the different organs, and particularly between blood and these organs, is a problem fraught with many difficulties, for besides those already alluded to, it is essential that the blood-vessels of the organ or tissue be thoroughly washed free of blood by perfusion before the extract is made, since blood itself contains considerable quantities of glycogenase. It is only when great differences are observed to occur that any conclusions can be drawn. As an example of the general distribution of glycogenase in different organs of the dog, the following results may be of interest :—

Organ.	Percentile Glyco-genolysis.	Duration of Incubation.	Remarks.
Pancreas	100·0	4 hours.	1·8 saline extract
Liver .	88·6	,,	Buchner extracts
Serum .	83·3	,,	
Kidney .	48·9	,,	
Muscle .	0·0	,,	

(MACLEOD.)

These results indicate that the pancreas is overwhelmingly richer in glycogenase than any other organ or tissue of the body ; it is present in the blood and liver, whereas the muscles, the heart, the kidneys, and the intestines contain it only in small amounts. In view of the recent work of Hill, Meyerhoff and others on the role of glycogen in the production of lactic acid during muscular contraction and the absence of glycogenase in muscle may explain why this acid rather than glucose is formed. Wohlgemuth and Benzur, however, assert that muscles contain diastase.

The evidence indicates that the glycogenase present in the blood is derived from the pancreas in normal animals, and is distributed to the various other organs and tissues in which it is found to occur. When the pancreas is absent it is possible that other organs may assume the function of producing glycogenase. One might have expected that glycogenase would be produced independently in the liver, since this is the chief seat of glycogenolysis, and, therefore, that glycogenase would be present in greater concentration in extracts of liver than in blood serum. Although, for reasons already indicated, it is extremely

difficult to arrive at any basis of comparison of the concentration of glycogenase in serum and liver, the following results indicate that there is relatively more in the serum :—

Animal.	Percentile Glycogenolysis in: Serum.	Liver.	Time of Incubation.	Remarks.
Dog	100·0	7·6	16½ hrs.	—
,,	60·0	55·1	16 ,,	Blood not washed out of liver.
,,	100·0	35·9 — 45·3	3½ ,,	Reaction varied.
,,	37·7	100·0	1¼ ,,	Starved dog.
,,	31·9	30·0	2 ,,	Overfed dog.
,,	100·0	58·2	5 ,,	——
,,	70·5	52·5	2 ,,	——
,,	57·4	7·2	2 ,,	——
,,	100·0	44·1	2 ,,	Starved dog.
,,	100·0	28·4	2 ,,	Ordinarily fed dog.
,,	63·8	42·8	3 ,,	——
,,	63·0	10·5	2 ,,	Exact time of incubation uncertain.
,,	64·5	17·9	2 ,,	——
Lamb	19·6	5·7	4 ,,	——
Pig	71·0	39·0	4 ,,	After standing four days.
Rabbit	72·3	34·2	4 ,,	——
,,	38·5	12·2	2 ,,	——

(Macleod and Pearce.)

Pick (1902), as well as Mendel and Saiki (1908), using alcohol powders, found the liver stronger than the serum, Pugliese and Domenichini (1907), who measured the amount of sugar which was produced instead of the glycogen which disappeared, also thought that the liver was richer in glycogenase than the blood serum. After allowing for the various sources of error, however, it appears that, in general, blood serum contains a higher concentration of glycogenase than an equal volume of liver. This raises the question as to whether the glycogenase which is found in the liver may not be that present in blood or lymph that has been incompletely removed from the tissues prior to preparing the extract. That such is not the case has been shown by finding that when the vessels of one lobe of the liver were washed out with isotonic saline for an hour, an extract prepared from this lobe had practically the same glycogenolytic activity as that of one prepared from the fresh organ just after removal of the blood. Evidently, therefore, glycogenase present in the liver cells, although it may be derived from the blood, remains fixed in them and is not removable by the perfusion of the blood-vessels with saline.

The possibility suggested by these observations, that the pancreas is the source of glycogenase, has been further tested by measuring the diastatic activity of the blood serum in animals

after pancreatectomy or ligation of the ducts of the gland.[1] With the exception of Schlesinger (1908), all of those who have attacked this problem are agreed that glycogenase still persists after complete pancreatectomy, although it may undergo variations in amount. Immediately following the operation a decided diminution, lasting in dogs for perhaps eight or ten days, may occur, after which, however, the amount recovers to a level decidedly lower than that of the normal, and remains constant until death (Otten and Galloway, 1910).

Other investigators, such as Carlson and Luckhardt (1909), could find no significant change, and yet others report a decided increase (Milne and Peters, 1912 ; V. C. Myers, etc.). The question is of interest because of the possible relationship of the blood and tissue glycogenases to diabetes, Cammidge and V. C. Myers, among others, having suggested that the hyperglycæmia in this disease is dependent upon increased activity of glycogenase in the liver, although Bainbridge and Beddard (1907) found in two cases suffering from this disease that no blood diastase could be demonstrated in the serum.

In view of these contradictory results, Markowitz and Hough have reinvestigated the problem in my laboratory. For determination of the diastatic power, the amount of reducing sugar formed in a unit of time in a mixture of well-buffered starch solution and blood plasma (Myers' method) was measured. Although there are possible objections to this method (p. 147), it was chosen because of its extensive use in clinical investigations. Pancreatectomy was usually found to cause a decided diminution in the diastatic power of the blood plasma in twenty-four hours after the operation. In a typical case, it fell in this time to about one-half its previous level, and then more gradually (in four days) to one-third, about which level it fluctuated slightly from day to day. Several months after pancreatectomy, in animals kept alive by insulin (p. 78), the blood diastase may return to the normal level. Expressing the diastatic power in terms of the milligrams of glucose formed in fifteen minutes, when the mixture contains 10 mg. of starch and 0·2 c.c. of blood serum, *less* the glucose originally present in this amount of serum,

[1] So far as we are aware, no one has conducted these observations by using glycogen instead of starch, but it is improbable that any serious error is hereby incurred, since standard conditions were maintained in the different observations.

it was found that 0·152 mg. was formed as the average of observations on sixteen normal dogs, the maximum being 0·255 and the minimum, 0·082 mg. In twenty-four hours after pancreatectomy it fell decidedly in five out of six animals, but rose in one. The following table shows results obtained several months after pancreatectomy :—

No.	Mg. Sugar Formed in 15 Minutes.	Plasma Sugar.	Remarks.
Dog. 20 .	0·088	0·338	Diabetic for seven months.
„ 23 .	0·058	0·380	Diabetic for six months.
„ „ .	0·113	0·077	„ „ twenty „
„ 32 .	0·083	0·150	„ „ two „
„ Tr. .	0·189	0·613	„ „ six „
„ Lu. .	0·141	0·593	„ „ four „

In a recently depancreatised animal administration of insulin usually raises the diastatic power of the serum, but not sufficiently to restore it to the normal level. Sometimes, on the other hand, the reverse result may be obtained, especially in partially depancreatised animals, or in completely depancreatised ones after some months. The results show clearly that the action of insulin is not related to the presence of diastase in the body. There is no experimental justification for the significance which has been given to variations in the diastase of the blood and urine in diabetes mellitus (Cammidge, Myers, etc.).

Increased activity of the pancreas, brought about by injecting secretin, does not cause an increase in blood diastase, but this occurs after ligation of all of the pancreatic ducts (Wohlgemuth, 1909). It is probably safe to conclude that although a certain amount of the glycogenase of blood serum is of pancreatic origin, some is also derived from other sources, and it is possible that the pancreatic diastase finds its way into the blood by being absorbed from the intestine.

The problem has also been attacked by comparing the concentrations of glycogenase in the blood of the pancreatico-duodenal vein, or in the lymph of the thoracic duct, with that of the femoral artery in anæsthetised dogs before, during, and following stimulation of the great splanchnic nerve. As explained elsewhere (p. 224), stimulation of this nerve leads to marked increase of hepatic glycogenolysis and hyperglycæmia. The results shown in Table XV. were obtained by Pearce and Macleod.

TABLE XV.

GLYCOGENASE IN THE BLOOD AND LYMPH AS AFFECTED BY STIMULATION OF THE SPLANCHNIC NERVE.

No. of Expt.	Nature and Amount of Fluid Used.	Experimental Condition.	Glycogen (dextrose) which Disappeared in Same Time.	Remarks.
4	Femoral artery	Before stimulating great splanchnic nerve . .	0·108	
,,	,, ,,	During 1 hr. stimulation .	0·108	
,,	,, ,,	30 min. after stimulating off	0·103	Incubated 3 hrs., 1 c.c. serum. Starch test gave dextrines in 60 mins. with all.
,,	Pancreatic duodenal vein	Before stimulation . .	0·103	
,,	,, ,,	During 1 hr. stimulation .	0·103	
,,	,, ,,	30 min. after stimulating off	0·105	
6	Femoral artery	Before stimulating great splanchnic nerve . .	0·074	
,,	,, ,,	During 30 min. stimulation	0·101	Incubated 4½ hrs. 1 c.c. serum in each case. Starch test gave dextrines first with.*
,,	,, ,,	45 min. after stimulating off	0·079	
,,	Pancreatic duodenal vein	Before stimulating . .	0·081	
,,	,, ,,	During 30 min. stimulation	0·082	
,,	,, ,,	45 min. after stimulating off	0·090	
,,	Lymph . .	Before stimulating . .	0·088	
,,	,, * . .	During 30 min. stimulation	0·115	2 c.c. serum incubated 3½ hrs.
,,	,, . .	45 min. after stimulating off	0·101	

In one of the experiments more glycogenase was found in the lymph collected during periods of stimulation, but this result cannot be given much weight as an indication of the pancreatic origin of the glycogenase, since it may have been due to curtailment of lymph flow, and in any case, most of the lymph is derived from the liver and only a small fraction of it from the pancreas. In another experiment this increase in the lymph was not observed to occur. Erhmann and Wohlgemuth (1909) were also unable to demonstrate that more diastatic enzyme is present in the blood of the portal vein than in that of the femoral artery.

Comparison of the diastatic activity of the lymph collected in various lymph vessels in the cat and dog by Carlson and Luckhardt, indicated that there is less in the lymph than in blood serum, and that increasing

the lymph-flow, by injecting lymphagogues, only sometimes causes the diastatic power to become greater. Röhmann and Bial (1894) also found the thoracic lymph of feebler diastatic power than the blood serum.

Taking these results as a whole, there is no evidence of a higher concentration of glycogenase in the vessels coming directly from the pancreas than in those from the rest of the body.

Comparison of the Concentration of Glycogenase in the Blood and Organs of Animals of Different Species.—If the amount of glycogenase bears an essential relationship to carbohydrate metabolism, it might be expected that it would be present in greater concentration in animals living on foods containing large quantities of carbohydrate than in those living on non-carbohydrate substances. Such comparisons are rendered difficult on account of the fact that both the blood serum and extracts of organs from animals of the same species show considerable variations in glycogenolytic power. Thus, in each of five dogs that were fed alike, percentile glycogenolysis, in similarly prepared extracts of the liver, amounted to 25, 25·4, 32, 43, and 58; and in 1 c.c. quantities of serum from the same animals, to 31·9, 25·5, 57·4, 63·0, 64·5, and 100. The differences, however, become much greater when animals of different species are used. Thus Pearce and I found that liver extracts of omnivorous animals, such as the dog, cat, or pig, contained in general much more glycogenase than those of herbivorous animals, such as the rabbit, sheep, or ox (see also Noel Paton and Maclean). These results would seem to indicate that the diastases of the blood and liver can be of no importance in the metabolism of preformed carbohydrates taken with the food, but why they should be most abundant in animals which depend mainly on proteins and fats cannot be said.

These observations suggested a comparison of diastase concentration in relationship to diet in animals of the same species. In extract from the liver of the dog, Macleod and Pearce found that 44 per cent. glycogenolysis occurred in four hours in preparations made from a starved animal, and only 30 per cent. in that of one that had been well fed.

Bang, Ljungdahl, and Bohm (1907) also found slightly more glycogenase in the liver of starved rabbits than in those of animals that were well fed. Since these comparisons were not made on the basis

of nitrogen or ash content, it is possible that the differences observed were due to greater dilution of the extracts by the large amounts of glycogen present in the livers of the well-fed animals. Bradley and Kellersberger (1913), using tissue powders prepared by the alcohol method, compared the diastase content and the glycogen in the liver, muscle, and blood of a large number of different sea animals, but were unable to demonstrate any relationship. Thus tissues rich in diastase might or might not contain glycogen, and also tissues rich in glycogen might contain no diastase. They consider it unlikely that the synthesis of glycogen in animals is dependent on the presence of glycogenase, thus contrasting with the condition found in plants, namely, that practically all the starch-storing tissues contain diastase during the developmental stages, as well as during sprouting. On the other hand, there are certain plant tissues that are rich in diastase and do not develop starch.

Post-mortem Glycogenolysis.—Very soon after death glycogen begins to disappear rapidly from the liver, and various investigators have studied the process with the hope that, by so doing, some light might be thrown on the factors which control sugar production by this viscus during life. It cannot be said that the results have been of any value for this purpose. The post-mortem process is apparently much more rapid than any that occurs during life. It is so rapid that no condition, associated with hyperglycæmia and glycogenolysis in the intact animal, can be shown to influence the rate in the liver when it is subsequently removed. Post-mortem glycogenolysis, in other words, always proceeds at the maximal rate uninfluenced by any ante-mortem condition. It will be unnecessary here to review in detail the evidence for this conclusion, and only brief reference will be made to some of the fundamental experiments.

It is difficult to tell exactly at what time after death glycogenolysis sets in. Considerable changes may occur before the liver can be removed from the animal, and it is significant that the concentration of sugar in the blood flowing from the liver undergoes a marked increase within a few minutes of interference with the blood flow. The process attains its maximum very rapidly, and then proceeds at a fairly constant intensity for some considerable time. This has been determined by keeping the excised liver at body temperature in the incubator and removing portions at hourly intervals for the determination of the glycogen. After five or six hours the process becomes accelerated, indicating the onset of putrefactive changes (see Macleod).

A most interesting question concerns the cause for the onset of post-mortem glycogenolysis. This may be, either that glycogenase increases in amount, or that, without such a change, conditions develop which are favourable to its action. By comparing the amounts of glycogenase, in an ice-cold saline extract of the liver immediately after removal, with that of a similarly prepared extract made from the same liver after it had been kept at body temperature for some time, no difference could be detected.

There can be little doubt that the onset of the process is dependent on the production of acid. Pavy and Bywaters (1910) found, for example, that alcoholic extracts of liver that had stood for some time contained much more acid than those prepared from liver that had been scalded or frozen immediately after death. They also observed that injection of sodium carbonate solution into the portal vein at the time of death prevented post-mortem glycogenolysis which, however, immediately started up by washing out the sodium carbonate from the liver. The two facts, namely, that the action of glycogenase is greatly affected by changes in reaction and that acid quickly forms in the liver after death, leave little doubt that the two are co-related. In view of the great speed with which it proceeds, it is improbable that studies of the post-mortem process can throw light on glycogenolysis occurring during life, although it is conceivable that changes in reaction in the liver cells may be an important part of the mechanism by which the *ante-mortem* process is controlled.

Observations of the rate of glycogenolysis in the liver *in situ*, after depriving it of its blood supply, before and during stimulation of the splanchnic nerve, have not revealed any differences (Macleod and Pearce). This result does not, however, indicate that glycogenolysis is uninfluenced through this nerve in the intact animal, for the result, no doubt, depends on the fact that the intensity of the glycogenolysis that is set up in the liver by curtailment of its blood supply is as great as possible under any circumstances, so that nervous impulses can make no impression. Mechanical interference with the portal blood flow, as by temporarily clamping it in anæsthetised animals, is soon followed by hyperglycæmia, but this does not occur after anastomosis of the portal vein with the vena cava, provided that the animals have been allowed to recover from the immediate effects of the operation. Complete detachment of the liver from the circulation, on the other hand, soon leads to hypoglycæmia (p. 266). Moderate degrees of disturbance in portal circulation also do not excite glycogenolysis; it has not been observed, for example, in patients suffering from thrombosis of the portal vein or, so far as is known, in other clinical cases of portal obstruction.

It will be evident from these facts that little information regarding ante-mortem glycogenolysis can be obtained from a study of the post-mortem process.

Attempts have been made to follow the behaviour of the glycogen in the liver during life, by determining the amounts in portions removed at intervals. When this is done in anæsthetised animals, extremely variable results are obtained. Glycogen invariably disappears, but does so decidedly more rapidly in the deeper lobes, as compared with the superficial ones. Differences in blood supply in the different portions, coupled with unavoidable chilling of the more superficial lobes, is probably responsible. Thus Macleod and Pearce found that variability in the amount of blood left in the viscus had a very pronounced influence on the rate of disappearance of glycogen. It is unlikely that any useful information can be obtained by observations of this type conducted on anæsthetised animals. Recognising this fact, Cori, Cori and Pucher have devised a surgical procedure by which a permanent opening is made in the abdominal wall in rabbits. This opening can be closed by a "window," and opened at intervals for the removal of portions of liver (about 1 gm. each). They have found that during the ingestion of sugar the concentration of free sugar in the liver and blood increases, accompanied also by an increase in glycogen in the liver as evaluated from the difference between the total carbohydrate and the free-sugar found present in it (see also p. 317).

The common assumption that glycogen is primarily a storage form of carbohydrate in the animal, as starch is in the plant, is not adequate to explain all the facts which are known about it. It is undoubtedly of much greater significance in metabolism than a storage material. As has recently been pointed out by Cramer, Claude Bernard considered glycogen as an internal secretion, that is, as a product of the activity of the liver cells, which is discharged into the blood as glucose, in order to maintain the blood sugar level above a certain minimum. Bernard considered this glycogenic function as being independent of the supply of preformed carbohydrate in the food. In the strict sense, the term glycogenic should be restricted to the formation of glycogen from protein (and fat) and its breakdown to sugar,

rather than to the polymerisation of sugar itself into that substance. Hormones such as thyroxin and epinephrin stimulate this dynamically-acting glycogenic function, so that glycogen fails to be deposited in the liver, although excessive metabolism of carbohydrate may be proceeding in the organism as a whole. Cramer interprets many known facts of carbohydrate metabolism in light of this non-storage function of glycogen, and considers that insulin acts partly at least by inhibiting glycogen formation from protein (and fat). In support of this view he points to cytological changes in the liver cells following injection of excess of insulin, but he fails to take regard to the rapid disappearance of sugar when insulin is injected into eviscerated animals, or is added to the nutrient fluid of the isolated heart. Nevertheless, Cramer's paper is worth careful perusal.

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CHAPTER XII.

THE BEHAVIOUR OF GLYCOGEN FOLLOWING INSULIN.

In depancreatised dogs, as we have seen (p. 101), glycogen becomes abundantly deposited in the liver when insulin is given along with sugar. This result led us to expect that the same process would also be readily demonstrable in normal animals injected with insulin, and thus account for the disappearance of free sugar from the blood. Much to our surprise, however, no increased glycogen deposition could be demonstrated to occur (McCormick and Macleod, 1923), and it became necessary to extend the scope of the investigations so as to make certain that the considerable variability in the amount of glycogen, which, even under the most carefully controlled conditions, is the rule in these animals (p. 140), was not masking the process. The investigations were therefore extended in scope, so as to include the following :—

(1) Comparison of the glycogen content of the liver, heart, and muscles of previously starved rabbits to which sugar was given either with or without insulin.

(2) Comparison of the extent of glycogen formation in the perfused liver of the turtle when insulin was present in the nutrient solution, with that occurring in its absence.

(3) Observations of the effect of insulin on the process of post-mortem glycogenolysis

The effect of insulin on fed animals was simultaneously being investigated by Dudley and Marrian.

(1) **The Influence of Insulin on Glycogen Formation in Normal Rabbits.**—Food was withheld from six rabbits of uniform weight for three days, each animal being also injected on the second or third day with epinephrin, so as to reduce the glycogen content as far as possible (p. 228). Certain of the rabbits in each batch were then given carbohydrate, the exact amounts varying in different experiments, although every rabbit of a particular

batch received exactly the same quantities. Insulin was given to certain of the rabbits in amounts just insufficient to cause convulsions, and others were used as controls. In several hours (three to six) all the animals were killed, the liver, leg muscles, and heart quickly excised, and the glycogen determined in them, great care being taken to keep the details of analysis constant for the various specimens of each series (McCormick and Macleod, 1923).

In the first observations equal amounts of sugar were injected subcutaneously every half hour into each of seven rabbits, and insulin also into four of them. Two other rabbits were injected with physiological saline, instead of sugar, and one of these also received insulin. In four and in six hours, animals from each group were killed. It was found: (1) that there were only traces of glycogen in the muscles (0·01-0·05 per cent.) and liver (0·01-0·30 per cent.) of the animals injected with saline, there being, perhaps, somewhat less in those which had received insulin; (2) that much glycogen had become formed in the livers (0·5-6·0 per cent.) of the sugar-injected animals, there being no difference between normal animals and those treated with insulin; (3) that more glycogen had become deposited in the hearts of the insulin-treated animals. This experiment was repeated with the modification that insulin was more frequently injected. The results of these experiments are shown in Table XVI:—

TABLE XVI.

	Normal Rabbits.			Insulin Rabbits.		
	Per Cent. Glycogen:			Per Cent. Glycogen:		
	Heart.	Muscle.	Liver.	Heart.	Muscle.	Liver.
Dextrose, 4 hrs. . .	0·35	0·10	4·16	0·40	0·17	1·48
Dextrose, 4 hrs. 40 min.	—	—	—	0·64	0·12	3·88
Dextrose, 6 hrs. . .	0·41	0·09	5·56	0·83	lost.	5·96
Saline, 6 hrs. . .	0·62	0·05	0·30	0·21	0·01	0·01 (*)
Dextrose, 4 hrs. . .	0·44	0·01	3·44	0·44	0·06	3·72
Dextrose, 6 hrs. . .	0·43	0·12	8·70	0·44	0·10	3·08 (*)
Saline, 4 hrs. . .	lost	0·01	0·26	0·06	0·02	0·14 (C)
Saline, 6 hrs. . .	0·13	0·05	0 08	0·41	0·13	0·55 (C)
Dextrose, 4 hrs. . .	0·44	0·01	4·76	0·82	0·02	2·60 (*)
Dextrose, 6 hrs. . .	—	—	—	1·11	0·08	3·56
Dextrose, 6 hrs. . .	0·70	0·03	5·12	0·84	0·20	2·92
Saline, 2 hrs. 50 min. .	0·18	0·004	0·52	0·09	0·005	0·24 (*)

(C) Indicates convulsions. (*) Death during convulsions or while in coma.

It can be seen that, under the conditions of the experiments, insulin has no constant influence on glycogen deposition in skeletal muscle, that it tends to accelerate this in the heart, but

to retard it in the liver. Thus, in four hours, the average amount of glycogen in the liver of the insulin-treated animals (including the one at four hours forty minutes) was 2·92 per cent., whereas in the controls it was 4·09 per cent.; in six hours these values, respectively, were 3·88 per cent. and 6·46 per cent. In two of the insulin cases, notwithstanding that sugar was given, convulsions occurred, although 2·6 and 3·08 per cent. of glycogen was found in the livers.

Since very large amounts of sugar were being assimilated, in the foregoing experiments, it was considered possible that the formation of glycogen might have been proceeding at the optimal rate, even in the absence of an excess of insulin. The procedure was therefore modified, either by giving the sugar by stomach tube and killing the animals after from two to six hours, or by feeding with measured quantities of oats for several days. The results with the glucose experiments were inconstant; those with oats were as follows :—

(All animals fed similar quantities of oats for five days following a period of starvation.)

Animals given Insulin Daily.		*Animals not given Insulin.*	
1. Liver glycogen,	2·15 per cent. (C)	1 Liver glycogen,	3·0 per cent.
2. ,,	0·27 ,,	2. ,,	3·65 ,,
3. ,,	0·028 ,, (*)	3. ,,	0·74 ,,
		4. ,,	1·5 ,,

(C) Indicates convulsions. (*) Death during convulsions or while in coma.

Decidedly less glycogen was present in the insulin-treated animals, and although in one of them fatal convulsions may be held accountable, these did not occur in the other cases.

In a repetition of this experiment, in which the animals were closely observed during a feeding period of seventy-five hours, and in which insulin was given to certain of the animals in sufficient amounts to cause persistent hypoglycæmia for the second half of the period (as determined by actual blood sugar determinations), the following results were obtained :—

No.	Normal Rabbits. Per Cent. Glycogen in:			No.	Insulin Rabbits. Per Cent. Glycogen in:		
	Heart.	Muscle.	Liver.		Heart.	Muscle.	Liver.
6	0·22	0·27	2·18	1	0·32	0·10	1·60 (*)
7	—	0·15	1·18	2	0·32	0·32	0·77 (C)
8	0·11	0·15	0·43	3	0·49	0·14	2·25 (C)
—	—	—	—	4	0·30	0·22	2·08
—	—	—	—	5	0·07	0·10	2·26

(C) Indicates convulsions. (*) Death during convulsions or while in coma.

It is clear that there is no essential difference in the glycogen of the liver or muscles, though there is usually somewhat more in the heart of the insulin-treated animals.

Taking the results as a whole, there is no evidence that injection of insulin into normal animals causes any significant change in glycogen formation, except possibly that it causes more glycogen to be deposited in the heart. In so far as the liver is concerned, insulin injections usually cause less glycogen to be deposited than in control animals given the same amount of carbohydrate. This is no doubt dependent on the mobilisation of sugar from the glycogen stores to replace that disappearing into the tissues (see p. 272).

At the same time as these observations were in progress, Dudley and Marrian (1923) were attacking the problem from a somewhat different standpoint. Instead of using animals from which as much glycogen had been removed as possible, they used well-fed ones, and injected sufficient insulin to bring the blood sugar to the convulsive level. Thus, two rabbits were fed during two days with carbohydrate-rich food, and one of them was then given insulin. Convulsions developed in six hours, when both animals were killed, and the following amounts of glycogen found :—

	Normal Rabbit.	Insulin Rabbit.
Liver	5·53	1·86
Heart	0·26	0·54
Skeletal muscle . .	0·57	0·0

The important fact brought to light by these investigations is the reduction to vanishing point of the glycogen of the muscles, and since the rabbit was killed at the time convulsions appeared, and not some time later, it does not seem probable that the reduction can be accounted for, solely, as the result of the convulsions. There must have been some reduction before these supervened, although probably the finishing touches to the disappearance were given by the convulsive symptoms. The same workers also compared the amount of glycogen in the livers of well-fed mice injected with insulin with that of uninjected mice, with similar results.

Dudley and Marrian's experiments have recently been repeated by Chaikoff in my laboratory.

Eight rabbits of approximately the same weights were fed on carrots and sugar for twenty-four hours. All were given 10 units of insulin (about 10.30 A.M.), and an hour later one was killed while the others received 10 more units of insulin. At the end of the second hour a second rabbit was killed and insulin injected into the others. This was continued until 50 units had been injected into each rabbit, or until convulsions supervened, when the animals were killed, except in one case, when the convulsions were allowed to continue for thirty-five minutes.

The following percentage amounts of glycogen were found :—

Units Insulin Injected.	Condition.	Glycogen (per Cent.): Muscles.	Liver.
10	Killed in 1 hour	0·22	2·4
20	,, ,, 2 hours	0·61	—
30	,, ,, 3 ,, ...	0·44	3·82
30	,, when convulsions appeared, 1.45 P.M.	0·31	4·75
30	,, 35 minutes after convulsions . .	0·19	2·52
50	,, when convulsions appeared, 3.50 P.M.	0·42	4·67
50	,, when convulsions appeared, 3.35 P.M.	0·32	1·92

The experiment was repeated, both on starved and fed rabbits, with the difference that the hypoglycæmic symptoms were allowed to continue for as long as possible.

Nutritive Condition.	No. of Animals in Each Group.	Duration of Symptoms.	Condition at Death.	Per Cent. Glycogen: Muscles.	Liver.
Starved 1 day	1	Short	Died after convulsions	0·010	—
	2	,,	Died after convulsions	0·016	3
	3	,,	Died after convulsions	0·106	—
Starved 2 days	1	1 hr. 10 min.	Died in convulsions	0·033	0·64
	2	3 ,, 30 ,,	Killed in coma	0·003	3·72
	3	1 ,, 35 ,,	Died ,, ,,	0·007	0·84
	4	2 ,, 10 ,,	,, ,, ,,	0·004	1·07
Fed carrots and sugar	1	3 ,, 10 ,,	Killed in coma	0·068	3·75
	2	5 ,, 30 ,,	Killed	0·11	4·20
	3	1 ,, 45 ,,	Died in convulsions	None	3·1
Fed carrots and sugar	1	3 ,, 0 ,,	Killed while still in good condition	0·79	5·4
	2	2 ,, 40 ,,		0·26	3·0
	3	4 ,, 26 ,,		0·53	3·0

In all animals which died as a result of the hypoglycæmic symptoms glycogen had practically disappeared from the muscles, although a fair amount still remained in the liver. In those killed while still in good condition, although symptoms had been pronounced for some time, no reduction in glycogen was evident. The animals of this last group were all richly fed.

It seems clear that there is no significant relationship between the incidence of symptoms and the percentage of glycogen in the muscles. When for some unknown reason this glycogen becomes greatly reduced in amount, the convulsive type of symptoms gives place to the comatose, and death soon follows. When glycogen remains, on the other hand, the animal may continue to show violent convulsions for several hours. The muscular contractions, and possibly also the respiratory embarrassment occurring during the convulsions, lead to disappearance of the muscle glycogen, and the loss, in animals which survive, must be immediately replaced from the liver reserves. It is significant that as much glycogen may remain in the liver of animals in which little remains in the muscles, as in those with about normal amounts. We shall see later (p. 176) that excess of insulin in the body inhibits the hyperglycogenolysis caused by asphyxia, etc., and it is possible that it is on this account that the liver stores were not used in Chaikoff's experiments.

Babkin has also investigated the influence of insulin on glycogen formation in rabbits, and has found that there is no pronounced accumulation of this substance in the liver and skeletal muscles when glucose and insulin are given simultaneously. In previously well-fed animals, on the other hand, insulin causes decided reduction of glycogen in both these tissues. Brugsch (1924), Nitzescu (1923), and Gigon and Staub (1924) have also found that insulin reduces the glycogen of the liver in well-fed rabbits.

These results are in striking contrast to those obtained by feeding glucose to diabetic animals injected with insulin, and we conclude that the difference is due to the fact that normal animals can at all times release from the pancreas a sufficient supply of insulin to metabolise, or polymerise, whatever amounts of carbohydrate may be present in the body. In other words, the endogenous supply of insulin is always at an optimum in the normal animal, so that the addition of more does not have any

effect in causing increased glycogen formation. In the diabetic organism, on the other hand, no insulin being available from endogenous sources, its injection immediately stimulates this process. There is no evidence in these investigations that the rapid disappearance of blood sugar caused by insulin in normal animals is due to glycogen formation.

Cori, Cori and Pucher (1923) have determined the free sugar (water extraction) and the glycogen in portions of liver removed at intervals from the liver through the abdominal window referred to elsewhere (p. 159).

Following the injection of 5 gms. of glucose into the stomach, the free sugar of the liver rose from an initial value of 0·35 per cent. to 0·43 per cent. in thirty minutes, and the glycogen from 1·5 per cent. to 1·8 per cent., these values in thirty-three minutes later being 0·49 per cent. and 2·0 per cent. respectively. (This means that 0·3 gm. of glycogen per 100 gms. liver were formed within thirty minutes.) Next day, after twelve hours' starvation, the same animal was found to have 0·300 per cent. free sugar and 1·43 per cent. glycogen in the liver twenty minutes after an injection of 2 units insulin, and after 5 gms. glucose, given by stomach along with 2 units more of insulin, the free sugar did not rise above 0·32, although the glycogen rose to 1·8 per cent. within one hour. Similar results were obtained in other observations of the same type, and it was concluded that glycogen is not formed in the normal animal unless the concentration of free sugar is above 0·30 per cent., but that it occurs below this level during the action of insulin. In view of the well-known variations which may arise in the glycogen distribution over the liver, especially when there is any disturbance of the organ (p. 142), and of the possibility of reflex and asphyxial disturbances, it would be desirable to have more data on animals not given insulin, and also to devise some method by which the glycogen could be determined directly, instead of indirectly, as was apparently the case in these observations. The methods used for measurement of the free sugar are also unsatisfactory.

At a later date Cori found in rabbits, mice, and guinea-pigs that insulin causes the liver glycogen to become diminished when a large amount is present to start with, but not so when this amount is small. After starvation the average glycogen of the liver, in sixteen mice injected with sub-convulsive doses of insulin, was 39 per cent. lower than that of the corresponding sixteen control mice. Since the quantities of tissue available for analysis in the animals were very small, the amount of glycogen was not determined directly, but was calculated from the difference between the total reducing power of the tissue after

its hydrolysis by means of acid, and that of simple watery extracts. In some preliminary experiments it was shown, by comparison of the glycogen as determined by Pflüger's process and that calculated by difference, that the latter is about 5 per cent. greater in normal animals and about 10 per cent. in insulin-treated ones. This fact seems to us to be of considerable significance. In some of the observations on rabbits the glycogen was determined directly with similar results.

Attention is called to the interesting fact that insulin may cause reduction in both glycogen and free sugar in the liver, whereas epinephrin, while lowering the glycogen, causes the free sugar to increase. It is supposed that this must indicate that the sugar which disappears under insulin is oxidised, the energy thus liberated being used to bring about synthesis of glycogen, such, for example, as occurs in muscles following contraction. In support of this interesting hypothesis, observations were made on the effect of insulin on glycogen formation in the liver of phlorhizined and depancreatised animals (cats and rabbits) previously starved, with the result that glycogen was found to be deposited. In these cases the insulin had formed glycogen from sugar derived from endogenous sources, since no food was given the animals after insulin was started.

Reference may be made here to the work of Bissinger *et al.* (p. 301), in which it was shown, in white mice, that when glucose (50 mg.) was injected along with insulin it disappeared three times more quickly (as determined by extracting the entire animal), and caused, in half an hour, three times as much glycogen to be formed in the liver as when sugar was injected alone. According to Lesser (1924), under whose direction this important work was done, insulin stimulates glycogen formation at the same time as it excites increased oxidation of glucose, the two processes running parallel, so that the sugar vacuum in the tissues—the glucatonia—which is responsible for the hypoglycæmia is the result. These observers also found that in one hour after the injection of sugar and insulin *all* of the injected sugar had disappeared, and the glycogen which was temporarily increased had returned to the hunger value. Now, it is clear that this sugar cannot have disappeared because of increased oxidation (see p. 256), so that one must conclude that some intermediary product of sugar metabolism was formed. This is apparently not a substance extractable by the fat solvents, since Dudley and Marrian found that the fat content of the liver (and

also the I-value of the fat) was the same in insulin-treated as in normal mice. One must remember, however, that the determination of fats is subject to considerable sources of inaccuracy.

According, therefore, to the observations of Cori, Cori and Pucher (1923), and of Bissinger, Lesser, and Zipf (1923), glycogen is formed at an *early* stage of insulin action, whereas, according to the observations of McCormick and Macleod, as well as of Bissinger, there is no evidence that any excess remains later. Its formation, if such occurs, can only be temporary, and any that is formed must soon become broken down to compensate for the glucatonia which continues to be created in the tissues, provided there still remains excess of insulin in them.

As a matter of fact, there can be no doubt, as the observations of Dudley and Marrian show, that glycogenolysis both in the muscles and in the liver becomes stimulated during the hypoglycaemia due to insulin. The very close relationship which is known to exist between the amount of glycogen in the liver and the rate of recovery of blood sugar following insulin (p. 272) proves that increased hepatic glycogenolysis is the most important factor involved in the restoration to normal levels of the depressed blood sugar. When this process fails, either because of lack of glycogen or because the nerve pathway through which the impulses necessary to excite the process is severed, as occurs after the injection of ergotamin (Burn, 1923), then the blood sugar fails to recover at the usual rate.

(2) **The Influence of Insulin on the Glycogenic Function of the Perfused Liver of the Turtle.**—As explained elsewhere (p. 144), the liver in this animal consists of two lobes, each supplied by a branch of the so-called umbilical vein.

Two types of experiment were performed. In one, the glycogen formed in one lobe through which saline solution containing insulin and sugar had been perfused for some time, was compared with that found present in the other lobe, which was either meanwhile perfused with the same saline-sugar solution not containing insulin, or was removed prior to the perfusion. In the other type, the method of Snyder, Martin, and Levin (1922) was adopted, namely, to observe the amount of sugar in the fluid perfused through the liver in a unit of time, with or without insulin, great care being taken to avoid errors due to changes in volume flow or in pH.

In the following experiments both lobes were perfused with saline solution, insulin being added on the side indicated by the asterisks :—

Glycogen Per Cent.		Percentile Difference.	Duration of Perfusion. Hrs.	Per Cent. Glucose in Perfusion Fluid.
Right Lobe.	Left Lobe.			
1·55	1·28*	18	7½	0·5
2·72	2·54*	6·6	7½	0·5
3·42	3·20*	6·4	9¾	1·0
1·84*	1·43	22·3	10	0·2

Since the right lobe normally contains between 3·4 and 14·6 per cent. more glycogen than the left, without any perfusion (p. 144), it is clear that these results do not indicate that insulin has any glycogenic influence, for although there is some indication of this in the last experiment of the table, the result cannot be considered as significant in face of the otherwise consistently negative ones.

In observing the effect of insulin on the sugar concentration of the perfusate, a solution containing no sugar was perfused several times through the liver. In this way the sugar concentration steadily increased because of post-mortem glycogenolysis. Insulin was then added to the perfusion fluid, without finding that it had any influence on the rate at which sugar accumulated. This was confirmed in another series of observations, in which the procedure was different to the extent that the perfusate was not recirculated through the liver, but was collected during each ten minutes, the total amount of sugar determined and the results obtained for each portion added to those of the preceding portions.

The results of these experiments are shown in the curves of Fig. 16, in which the thin lines give the rate of sugar increase without insulin, and the thick ones, that after insulin was added to the perfusion fluid (Noble). Although they consistently show that insulin has no retarding influence on the process of post-mortem glycogenolysis in the turtle liver, and that it does not accelerate the rate of glycogen formation when the perfusate contains sugar, this does not necessarily mean that it has no influence on these processes, for it is now well established, though unknown when most of these experiments were performed, that it takes several days, instead of hours, for this hormone to cause hypoglycæmia in cold-blooded animals (p. 281). The observations are nevertheless of value, since they show that glycogen formation is not immediately accelerated in cold-blooded animals by the presence of insulin.

Observations in which allowance is made for this delayed action of insulin in cold-blooded animals have been recorded by Issekutz (1924), who used frogs injected with insulin (in the

months between November and February) fifteen to twenty-three hours prior to making the perfusions of the liver. He found that sugar was formed at the rate of 2·59 mg. per gram liver and hour in the case of normal frogs (with Ringer's solution), but that this increased to 5·39 mg. when epinephrin was added. Neither of

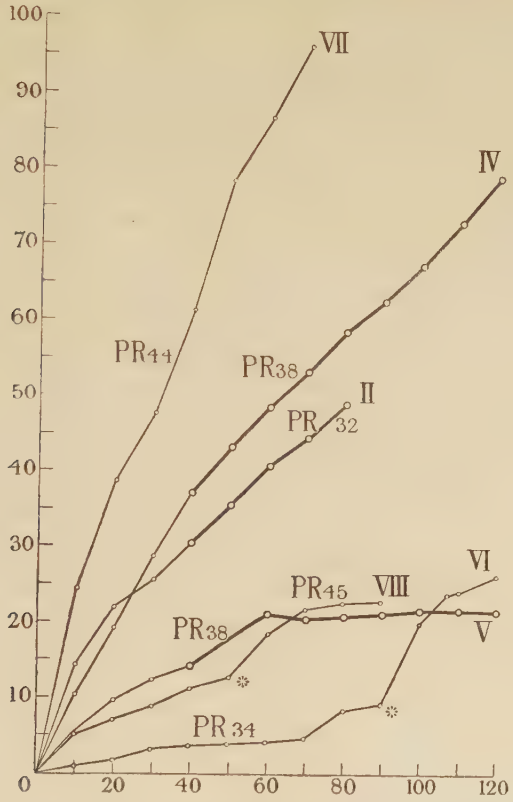

FIG. 16.—The percentage of sugar in artificial plasma perfused once through the liver of the turtle. The curves show the amounts of sugar found in the fluid collected during each ten-minute period of perfusion, the amount for each period being super-added. Ordinates, p.c. sugar. Abscissæ, time in minutes. P.R. indicates the rate of perfusion in c.c. per ten minutes. Insulin was added to the perfusion fluid during the periods indicated by the thick line curves. (From Noble and Macleod.)

these results was altered by adding insulin to the perfusion fluid, but when the livers of frogs previously injected with insulin were used, the rate of sugar formation without epinephrin was reduced to 0·53 mg., and with this hormone to 0·39 mg. These conspicuously lesser values obtained on the livers of

insulin-treated frogs were not due to the fact that they contained less glycogen than those of the normal animals before the perfusion was started. In view of the carefully controlled experiments of Snyder, Martin, and Levin (1922), showing that the main factor upon which the production of sugar in the perfused cold-blooded liver depends is the volume flow of the perfusate, coupled with its reaction, it is unfortunate that this was not more carefully controlled in Issekutz's experiments.

(3) **The Influence of Insulin on Post-mortem Glycogenolysis in the Mammalian Liver.**—The effect of insulin on this process was studied at an early stage in our investigations and since the results have not previously been published, the essential details will be given here:—

The following experiment was done with extract prepared by Banting and Best from ox pancreas. It was found to be active on depancreatised dogs, but to lose its potency on boiling. The liver of a richly-fed rabbit was passed through a chilled mincer. The mince was mixed with about an equal volume of defibrinated blood from the same animal and then divided into two equal portions, which were placed in flasks. To one of these, A, extract was added, and to the other, B, an equal volume of boiled extract. Portions were then removed from each flask for determination of glycogen, the flasks placed in the incubator and further portions removed at intervals.

Time of Incubation. (Min.)	Per Cent. of Original Glycogen Disappeared (Percentile Glycogenolysis).	
	A. (Insulin.)	B. (Control.)
0	0	0
30	90	84
90	91·4	(96·8)
150	100	93

There was, therefore, evidence that glycogenolysis had proceeded somewhat more rapidly in the presence of the unboiled extract. The process in both flasks was, however, so rapid that the result was not considered significant. This experiment was repeated with the difference that a considerable amount of phosphate buffer (Na_2HPO_4) was added to the mixture of Ringer's solution and blood, the control containing no insulin. After three hours' incubation there was 40 per cent. glycogenolysis in the presence of insulin and 50 per cent. in the control. The insulin, apparently, had a slight restraining influence on the glycogenolysis, but this was not considered significant for the reasons given elsewhere (p. 157).

The experiment was then modified in that the insulin—a very active preparation supplied by Collip—was injected into a rabbit, and one hour later a second quantity of the same extract was injected into the portal vein immediately after which the animal was killed, and the liver quickly excised. From this liver, and from one of a control animal, portions were then taken for determination of glycogen, and the livers placed side by side in an incubator. The following were the results :—

Time after Killing. Min.	Per Cent. Glycogen Remaining in Flasks.		Per Cent. Glycogen Disappeared (Percentile Glycogenolysis).	
	Insulin.	Normal.	Insulin.	Normal.
0	12	6·07	—	—
68	9·85	—	18	—
117	—	4·67	—	23
121	9·22	—	23	—
151	—	4·49	—	26
154	8·88	—	27·5	—

Here, again, no significant difference could be detected in the rate of glycogenolysis in the insulin treated as compared with the normal liver. Collazo (1924) has also found that insulin does not influence the rate of glycogenolysis in the liver of the guinea-pig.

Diastatic Action.—These results lead naturally to the question whether insulin can influence the speed of diastatic action. This we investigated by the method devised by Wohlgemuth with entirely negative results, that is to say, the starch-iodine colour reaction disappeared at exactly the same rate in buffered mixtures of soluble starch and saliva whether insulin was present or absent. Incidentally, in the course of these observations, it was also seen that insulin itself has no diastatic action.

Observations of a similar type have more recently been reported by Brugsch, Benatt, Horsters, and Katz (1924).

They compared the rate at which sugar and acid appear in incubated suspensions, in Ringer's solution, of ground-up liver and muscle removed immediately after death, either from normal or from insulin-injected animals. In suspensions of normal frog liver in 26 parts of Ringer solution the average number of mg. of sugar appearing per gramme of tissue per hour was 4·7, and the acidity, calculated as mg. lactic acid, 0·67, whereas these values in frogs injected with insulin (5 u.) two hours previous to killing, were 2·1 and 0·4 respectively, a

very decided decrease. Repetition of the observations with the liver of guinea-pigs, using in this case 3 parts liver to 25 parts Ringer's solution, gave per 10 gms. of liver, for starved animals, 71·0 mg. sugar and 2·4 mg. acid, and for starved animals injected with sufficient insulin to cause convulsions, only 19 mg. sugar and 2 mg. acid. These results indicate that insulin retards post-mortem glycogenolysis and they furnish evidence that it may encourage glycogen formation during life.

When glucose was added to liver emulsion from a starving animal injected with insulin, and oxygen was bubbled through the mixture, there was less glucose after one hour's incubation than at the start, and lactic acid did not accumulate in sufficient amount to account for the disappearing sugar. This is interpreted as indicating that an oxybiotic process occurs. When the liver of normal (starved or fed) animals was used, the glucose became increased in the absence of oxygen but decreased in its presence. The lactic acid in many of the experiments was only determined by titration of the acidity of the mixtures. Measurement of phosphoric acid showed a decrease accompanying the decrease of sugar in the mixtures containing the liver of insulin-injected animals.

The authors consider that their results demonstrate that the sugar which breaks down in the oxybiotic process unites with H_3PO_4 to form hexose-phosphate, which represents a step in a synthetic process resulting in the formation of glycogen. Insulin favours the oxybiotic process, so that in its absence, as in diabetes, this substance fails to be formed. Analogous studies on muscle are reported as furnishing similar results.

If the results of these somewhat complex experiments prove to be correct, they certainly indicate that insulin has some influence on the complex series of changes involved in the glycogenolytic and glycolytic processes, which go on side by side in a suspension of fresh liver tissue. The authors invoke the equations of Meyerhof, representing the relationship of glucose, phosphoric acid, and glycogen in muscles to explain their results. Chaikoff, working in my laboratory, has been unable to obtain similar results to Brugsch and his pupils.

The Influence of Insulin on the Glycogenolysis Occurring in Experimental Hyperglycæmia.—It is known that in experimental hyperglycæmia due to piqûre, epinephrin, or asphyxia, the glycogen rapidly disappears from the liver (p. 220), and that insulin can prevent the hyperglycæmia in each of these cases (p. 229). The question arises as to the mechanism hereby involved. Does the insulin act by retarding the glycogenolytic process—although by itself it rather accelerates it—or by causing the excess of sugar to be locked away as quickly as it is discharged into the blood from the liver? The fact that insulin can cause

injected glucose to disappear from the blood (p. 308) would favour the latter view, though not excluding the former. The following experiments were therefore performed in collaboration with Noble and O'Brien (1923):—

Each of a group of (usually six) well-fed rabbits, were injected with equal quantities of epinephrin (adrenalin) at regular intervals, extending usually over eight hours. Certain of the animals in each group were also injected, over the same periods, with as much insulin as they could tolerate without severe convulsive symptoms supervening. The animals were then killed and the glycogen determined in the livers. The results are shown in the following table:—

Date.	Per cent. Glycogen in Liver: Without Insulin.	With Insulin.	Duration of Experiment.	Remarks.
1923.				
March 29	1·40	8·12	8 hours.	———
,,	1·96	6·40	—	———
,,	—	3·46	—	———
April 12	1·19*	12·24	8 hours.	Died in three hours.*
,,	8·56	1·60*	—	Convulsions.*
,,	6·60	5·60	—	———
,, 19	2·30	3·34	8 hours.	———
,,	1·10	10·90*	—	Mild convulsions.*
,,	3·20	1·20*	—	Marked convulsions.*
Nov. 9	4·30	9·70	8 hours.	———
,,	8·00	10·20	—	———
Average	3·86	5·7		

It will be seen that there was, on an average, decidedly more glycogen in the livers of the animals treated with insulin than in those without. Two of the insulin-treated animals developed marked convulsions, and in them the livers contained low percentages of glycogen.

Notwithstanding every precaution to have the animals of the two groups treated alike with regard to feeding, there is always a considerable chance of error in experiments of this type. This is owing to the fact that one can never predict, in rabbits, the percentage of glycogen in the liver (p. 340).

In order to circumvent these sources of error we have more recently adopted the procedure of comparing the glycogen content of different portions of the same liver at the beginning and end of the experiment. The anæsthetic, ether or urethane, was in this case also the hyperglycæmia producing agency, comparison

of glycogen being therefore made between animals that were simply kept under the anæsthetic and others also injected at frequent intervals with insulin. The following experiments, conducted in collaboration with Miss O'Brien, will illustrate :—

A rabbit which had been well fed for several days was given 0·4 gm. urethane in solution intraperitoneally, and a piece of the right-central lobe of the liver was removed through a slit in the abdominal wall. It contained 8·64 per cent. glycogen. The animal was kept under urethane for eight hours, during which the rectal temperature remained tolerably constant. The blood sugar rose from 0·144-0·218 per cent. The liver was found to contain 3·52 per cent. glycogen at the end of the experiment.

In two other well-fed rabbits kept under urethane for periods of five to seven hours, the following values were found :—

1. Blood sugar rose from 0·114 per cent. to 0·330, and the liver glycogen after seven hours was 2·80 per cent.

2. Blood sugar rose from 0·127 to 0·310, and the liver glycogen after five hours was 0·69 per cent.

When insulin was also given in another rabbit the glycogen in a portion of liver removed at the start was 5·86 per cent., and the blood sugar fell to 0·103 per cent. The glycogen after seven hours was 5·76 per cent.

These results indicate that when sufficient insulin is given to prevent the development of hyperglycæmia, as a result of urethane, the glycogen of the liver is conserved.

One feature of the foregoing experiments which impressed us very much was the enormous quantity of insulin which could be injected, when epinephrin was also given, without the development of any symptoms. This led us to expect that a very extensive glycogenolysis must have been occurring, and that the sugar thus added to the blood was acting in the same way as injected sugar would have acted, but the results we have just considered show that this cannot have been the case, for instead of being augmented the glycogenolytic process was actually inhibited as a result of the insulin.

We may sum up the most definite results discussed in the present chapter as follows : Insulin given to previously starved animals along with carbohydrate does not significantly alter the rate at which glycogen is deposited—if anything, indeed, retarding it ; given to previously well-fed animals, it reduces the glycogen stores ; and given to animals in which hyperglycogenolysis is proceeding, as a result of injections of epinephrin or

anæsthetics, it retards this process. How may these results be harmonised among themselves, and with the fact that insulin, when given along with sugar to diabetic animals, stimulates glycogen formation? We believe by assuming that insulin stimulates the formation of some unknown substance which maintains a certain equilibrium with glycogen, and stands on the way between this and lactic acid. This unknown substance may be a stepping-stone in the conversion of carbohydrates into fats, and it is possible that in its formation a phosphoric acid complex, such as hexose phosphate, is involved (see p. 334).

Best, Hoet, and Marks have compared the percentages of glycogen in corresponding muscles removed from the hind legs in decapitated eviscerated cats before and after the continuous intravenous injection (for three to five hours) of glucose at the rate of 0·400 grm. per hour. Under these conditions, there was very little more glycogen after the perfusion than before. When, on the other hand, insulin was injected into the preparation as well as sugar at a rate sufficient to maintain the blood sugar at a high level, considerably more glycogen was found present. In six experiments of this type the increase in glycogen accounted for about 70 per cent. of the sugar which disappeared from the preparation as calculated by the method used by Burn and Dale, described on page 258. In one decapitated cat from which the viscera were not removed and in which insulin caused the blood sugar to fall to 0·05 per cent. the same percentages of glycogen were found at the beginning and end of the observation.

That glycogen is deposited in the muscles of an eviscerated (depancreatised) animal when there is excess both of insulin and sugar in the blood, parallels the observation that this also occurs under similar conditions in the liver of one that is merely depancreatised (p. 101). There is no doubt that under these conditions most of the sugar which disappears from the blood is converted to glycogen, but the question as to what happens to the sugar which leaves the blood when insulin alone is injected, still remains unanswered. As Lesser and his co-workers have shown (p. 302), it may be temporarily deposited in glycogen and this then gradually oxidised, but it seems more likely that it is converted into some non-carbohydrate substance or substances, since otherwise it is very difficult to explain why glycogen should tend to disappear in the presence of a large excess of

insulin. Under these conditions the evidence clearly shows that the glycogen of the liver becomes used up in an effort to restore the blood sugar to the normal level, but at the same time the glycogen of the muscles either remains constant (p. 164) or decreases somewhat. Meanwhile, in the rabbit at least, there is no evidence of increased carbohydrate combustion to account for the disappearing sugar.

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CHAPTER XIII.

THE SUGAR OF THE BLOOD.

THERE can be little doubt that the most useful single criterion of the behaviour of carbohydrate metabolism in the animal body is the percentage of sugar in the blood. This represents the balance between addition or new production of sugar, on the one hand, and loss of sugar on the other, and since each of these processes is dependent on a variety of factors, the significance of changes in the blood sugar can be appreciated only provided all of them are kept clearly in mind. Addition of glucose to the blood may be the result of absorption from the intestine, or of increased hydrolysis of glycogen (glycogenolysis) in the liver (or muscles), or of new production of glucose (gluconeogenesis), which may occur out of non-carbohydrate substances, such as the amino acids, and possibly fats. Removal of glucose may be the result of oxidation in the tissues or of excretion, mainly through the kidneys, or of polymerisation (glycogenesis), or of conversion into non-carbohydrate substances. When, in addition to these factors, it is remembered that the breakdown of glucose in the animal body proceeds through a series of intermediary products, it is evident that observations of the behaviour of glucose in the blood can furnish us with only fragmentary knowledge of the nature of this process as a whole. Notwithstanding these limitations, however, studies of blood sugar must form the most useful single means of attack on problems of carbohydrate metabolism. In the present chapter we will review briefly what is known regarding the amount, manner of combination, and the stability of the sugar in the blood.

BLOOD SUGAR UNDER VARIOUS NORMAL AND ABNORMAL CONDITIONS.

Methods for Estimating the Blood Sugar.—It would serve no useful purpose to review here in detail the various methods for the determination of the blood sugar. Several excellent ones

have been evolved in recent years, all of them fulfilling the requirements of simplicity, expedition, and accuracy. Since, also, only a small quantity, from 0·1-1 c.c., of blood is required, the observations can be frequently repeated without incurring serious errors due to loss of blood, even when comparatively small laboratory animals are used.

As the first step in all the methods, the laked blood is mixed with some reagent which removes the protein, so that, after filtration, a clear solution is obtained ; an aliquot portion of this is then mixed in alkaline reaction with some reagent which is capable of yielding oxygen to bring about oxidation of the aldehyde group of the sugar.[1] The reagent, therefore, becomes reduced into a lower oxidation product, and the extent to which this occurs can be readily measured by chemical means. In general, the methods can be divided into two groups, the titrimetric and the colorimetric. In the former, the extent of reduction of cupric to cuprous oxide is measured, either by titrating the latter by means of ferric sulphate and permanganate, as in Bertrand's method, or by iodometric principles, as in the methods of Bang, Maclean, Hagedorn-Jensen, and Shaffer-Hartmann (1921). In the colorimetric group, the development of a red colour due to reduction of picric acid to sodium picramate in the presence of sodium carbonate (S. R. Benedict's method), or of a blue colour, due to the action of cuprous oxide on Folin's reagent, is used.

The results of the different methods do not exactly correspond. In general, it may be stated that the colorimetric methods usually yield higher results than the titrimetric, and of the colorimetric methods, Benedict's usually yields higher results than that of Folin-Wu. The following table shows some comparisons made on samples of the same blood by Höst and Hatlehol :—

	Method.	Per Cent. Glucose.		
		1.	2.	3.
Titrimetric	Bang .	0·097	0·118	0·145
—	Hagedorn .	0·091	0·124	0 159
Colorimetric	Benedict .	0·118	0·151	0·198
—	Folin .	0·105	0·121	0·188

[1] For the removal of proteins the most successful methods are precipitation with colloidal iron, or picric acid, or sodium tungstate and sulphuric acid. When either of the last two reagents is used for this purpose the excess of it which passes into the filtrate may be made to serve as the reagent which becomes changed in colour by the reduction process.

The Shaffer-Hartmann method is particularly useful for routine purposes.

The Nature of the Sugar of the Blood and the Manner of its Combination therein.—In using the foregoing methods, it is assumed that the reducing power of the blood filtrate is a measure of the amount of glucose present. This assumption, however, is not entirely warranted, since the blood may contain traces of other reducing sugars, or of other substances having reducing powers, and among these may be mentioned glycuronic acid and creatinine. In order to determine the actual amount of glucose present, it has been customary to determine the difference in the reducing power before and after fermentation with yeast, any residual reduction being assumed to be due to other substances than glucose. In using this method, however, several possible sources of error must be borne in mind, for example, the yeast may itself contain reducing substances, or it may produce them during its action. Or, again, the yeast may so change the non-fermentable reducing substances as to rob them of their reducing qualities (Höst and Hatlehol, and Macleod). Quite apart, therefore, from the practical difficulty of making certain when fermentation of the glucose is complete, and that no other microbal action capable of destroying glucose has set in, the method of yeast fermentation is open to considerable sources of error.

Even if the reducing power after yeast fermentation could be measured with any degree of accuracy it would still leave us in the dark as to whether all of the reducing substance which had disappeared was glucose. It is possible also that among the residual-reducing substances might be included several of considerable importance in carbohydrate metabolism. For example, during its break-down, glucose may yield products such as dihydroxyacetone, acetaldehyde, etc., which have reducing properties but are unfermentable by yeast, and these may be intermediary products in its metabolism. In order to detect the presence of these substances, as well as of other possible intermediary products of carbohydrate metabolism, Stepp (1917, 1919) has introduced a method involving a new principle. The proteins and other colloids are first of all precipitated from the blood by phosphotungstic acid and, after removal of the excess of this reagent from the filtrate, the amount of carbon is determined in one portion and the reducing power in another. In many samples of blood the carbon, as calculated from the reducing sugar, has been found to correspond to that actually present, thus indicating that all of the reducing power of the blood

was due to glucose. In some cases, however, particularly in diabetes, the carbon actually found was less than that calculated from the reducing sugar, which would indicate that the blood contained reducing substances, with a lower percentage of carbon than glucose. It may be pointed out that if the amount of carbon were found to be greater than could be accounted for by the reducing power, it would indicate the presence either of sugars having a lower reducing power than glucose, or of a mixture of reducing substances with carbon compounds having no reducing properties.

Assuming that the great bulk, if not all, of the reducing substance in blood consists of glucose, there yet remains a possibility that some of it may be of a more reactive type than that met with in simple watery solutions of this sugar. As the result of the work of Irvine and his school, evidence has been brought forward to show that besides the α and β varieties of glucose, there probably also exists a third variety, styled γ-glucose, which differs from the others in exhibiting more marked reducing power, and in rotating the plane of polarised light to the left, instead of to the right. To explain these differences, it is assumed that in the ordinary varieties (α and β) of glucose the oxygen atom, in the ring form of the formula, is attached between the first and fourth (butylene) carbon atoms, whereas in this unstable (γ) variety the linkage occurs either with the second (ethylene) or the third (propylene) carbon atom. Based on these chemical considerations, it has been suggested by Hewitt and Pryde (1920) that during the absorption of glucose from the intestine, γ-glucose is formed, and is absorbed as such into the blood. To test this possibility, Hewitt and Pryde placed a hypotonic solution of glucose, after its rotating power had become constant, in a loop of intestine of an anæsthetised animal, and on removing it five minutes later, found that a change had taken place, making it less dextro-rotary. This change, however, was only transient, since in twenty-five minutes the solution after removal from the gut had reacquired its original rotating power. Neither Stiven and Waymouth Reid (1923), nor Eadie has been able to confirm these results, even in cases in which satisfactory clarification of the intestinal contents was effected within a few minutes of their removal. As has been pointed out by the former workers, it is unlikely, in the case that the unstable γ-glucose were formed, that any of it would remain in the fluid in the intestinal loop. It is much more likely that it would be

absorbed immediately into the blood. Moreover, as pointed out by Irvine, the reactive type of glucose is extremely unstable, and it is inconceivable that it could remain in the solution after its withdrawal from the gut.

Winter and Smith (1923) have presented evidence which they interpret as indicating the occurrence of γ-glucose in the normal blood of man and laboratory animals, but this evidence is not satisfactory.

It depends on the fact that after removal of proteins, first of all by Folin's reagent and subsequently by alcohol, the protein-free filtrate gives, with the polariscope, readings that are less dextro-rotary than they should be, as calculated from the reducing power (Shaffer-Hartmann method). Furthermore, they state that on standing *several days* the dextro-rotary power gradually rises to the calculated level. Eadie (1923), on repeating Winter and Smith's observations, by exactly the same methods as those recommended by them, has occasionally found, in blood from the normal rabbit and dog, results which at first sight might seem to be confirmatory of theirs. This can be seen by comparing the figures in columns three and four of Table XVII. (Eadie, 1923), (see next page).

We do not consider that these results offer any support to Winter and Smith's hypothesis. Such small differences in the readings as are observed may depend on the presence either of glucosides, or of lævo-rotary substances, and the changes may depend on gradual hydrolysis or destruction. These substances may, for example, be products of the long and complex chemical manipulations necessary to prepare the solutions for the polariscope, or they may be minute traces of protein. Moreover, the slowness of the change in rotating power scarcely suggests the presence of a *highly reactive substance.* These authors further state that in blood removed either from diabetic patients or from animals injected with epinephrin, no evidence can be obtained, by the above methods, of the presence of γ-glucose, but, on the contrary, that the polariscope readings of the blood filtrate in such cases are *more* dextro-rotary than those calculated from the reducing power. This, they interpret as indicating that blood contains complex sugars having a higher rotatory power, but a lower reducing power, than glucose. They conclude that insulin owes its beneficial effects in diabetes to the fact that it leads to a production of γ-glucose. Although some of the values

TABLE XVII.

1 Animal.	2 Blood Sugar mg. per 100 c.cm. when Sample Taken.	3 Readings on Successive Days. Degrees.	4 Reading Calculated from Reduction. Degrees.	5 * D of Lowest Reading. Degrees.	6 Vol. of Blood Taken. c.cm.	7 Time Required by Process. Hrs.
Rabbit, normal	—	0·07, 0·07, 0·17, 0·17.	0·17	21	100	6
„ „	—	0·08, 0·10, 0·07, 0·10.	0·17	25	85	6¾
Dog, normal	—	0·07, 0·15, 0·20.	0·21	18	100	9
„ „	—	0·23, 0·25, 0·30, 0·31, 0·31.	0·30	39	95	—
Rabbit, epinephrine	300	0·58, 0·55, 0·53, 0·53.	0·50	56	75	6¾
Dog, epinephrine	147	0·20, 0·20, 0·20.	0·21	47	100	10½
„ „	230	0·63, 0·61, 0·70, 0·70, 0·68.	0·70	48	100	—
Dog, epinephrine + insulin	300	0·08, 0·15, 0·28, 0·31, 0·28.	0·20	22	100	10¾
Dog, epinephrine + insulin	240	0·38, 0·50, 0·47.	0·50	39	100	—
Dog, epinephrine + insulin	145	0·17, 0·25, 0·23, 0·23.	0·13	66	100	5½
Dog, epinephrine + insulin	176	0·22, 0·16, 0·18, 0·16, 0·16.	0·20	42	100	6½

* For the calculation of rotating power from reducing (a) D = 52·5 was used.

given in the above table might be considered as favouring these views, there are just as many others which are contradictory.

Winter and Smith further postulate that an enzyme having the function of producing γ-glucose from the α-β form must exist in the body, and that this enzyme is activated by insulin. In support of this they state that the dextro-rotary power of solutions of glucose becomes diminished when acted upon by insulin in the presence of an extract of liver, a result which Eadie has been entirely unable to confirm. In a subsequent paper by these authors (1924) it is admitted that a reconsideration of the γ-glucose hypothesis is necessary. At the same time, however, these authors present further evidence for the presence of complex sugars in the blood of diabetic patients, which, in the light of work by others (see p. 191), may be of significance.

Lundsgaard and Hobell have recently published results of polariscopic examination of dialysates of blood, which they consider to give evidence of the presence of some sugar of lower

rotating power than α, β-glucose. Since actual polariscopic readings are not given, it is impossible to judge the value of the evidence. In any case, these readings must be very small, and subject, therefore, to large percentile error.

The possibility of combination between glucose and protein (amino acids) must be borne in mind when differences are found between the rotating and the reducing powers of blood or tissue extracts. Richaud and Coirre (cf. Grevenstuk and Laqueur), for example, found that the rotating power of a sterile mixture of certain sugars, or glucosides, and intestinal mucosa, or other tissues that had been treated with alcohol and heated to 120° C., gradually became less on incubation at body temperature, while the reducing power did so only to a slight degree. The lowest value was reached in one week, after which the rotating power rose again and returned to about the original in three weeks.

Distribution of Sugar between the Corpuscles and the Plasma.—Two methods are employed to investigate this problem—one, which we may call the *direct* method, consists in centrifuging the blood immediately after its removal from the body, pipetting off the serum or plasma, washing the residue with isotonic saline, and then determining the sugar separately in plasma and residue.

This method is entirely unsatisfactory for various reasons, chief of which are : (1) that it is difficult to precipitate all the proteins from the magma of corpuscles ; (2) that the washing of the corpuscles alters the sugar concentration within them because of diffusion into the wash-fluid ; and (3) because glycolysis (p. 194) occurs rapidly in the sediment, and leads to a considerable lowering of the sugar concentration.

A more satisfactory method, which we may call *indirect*, consists in measuring the sugar contained in a sample of whole blood, and in a sample of plasma obtained by rapid centrifuging.

At the same time, the proportion of corpuscles to plasma is determined by the hematocrite, and it is then an easy matter to calculate the concentration of sugar in the plasma and corpuscles. To circumvent the possibility that anti-coagulants, such as oxalates or citrates, might alter the permeability of the corpuscles, the blood should be received through a sterile cannula directly into a paraffined centrifuge tube, cooled and immediately centrifuged at a high speed. In a few minutes, enough plasma can then be secured for analysis. There is, however, no evidence to justify the statement made by Falta and Richter Quittner (1919), that such anti-coagulants do affect the permeability. (Cf. Ege, 1920, and Hagedorn, 1920).

Observations by the indirect method have been made with normal blood of various animals, and also with blood removed at various stages during changes in the sugar concentration due to disease, or to experimental conditions. The following table depicts some of the results that have been obtained :—

Animal.	Method Used for Measurement of Reducing Power.	Percentage Reducing Power:			Authors.
		Of Whole Blood.	Of Plasma.	Of Corpuscles (by Calculation).	
Man	Colorimetric	0·12	0·118	0·121	Bailey.
Dog	,,	0·062	0·079	0·026	—
		0·089	0·111	0·035	Wishart.
		0·096	0·100	0·087	—
Man	Schenck's method (Knapp's solution)	0·098	0·105	0·082	Tachau.
Dog	Bang	0·081	0·090	0·070	,,
Man	Colloidal iron and Bertrand	0·094	0·098	0·089	Rona and Döblin.
Man	Folin-Wu	0·090	0·096	0·081	Folin and Berglund.

It will be observed that, in the blood of man under normal conditions, the sugar concentration in corpuscles and plasma is about the same, there being sometimes a little more in the plasma than in the corpuscles. This difference is greater in the blood of the dog.

During hyperglycæmia, brought about by administration of glucose, the plasma may contain considerably more than the corpuscles, this difference being particularly marked while the hyperglycæmia is increasing; while it is declining, on the other hand, plasma and corpuscles show approximately the same amounts of sugar (Bailey, 1919; Wishart, 1920). Although these observations reveal a slight tendency to lag in the migration of sugar from the plasma into the corpuscles, they do not indicate that the corpuscles may act as temporary storehouses for glucose, since there is no lag in the opposite direction. The chief interest in such studies on blood is owing to the possibility that migration of sugar through the corpuscular membrane obeys the same laws as its migration into tissue cells in general.

In the blood of diabetic patients Frank (1911) found decidedly more sugar in the plasma than in the corpuscles, a fact which has also been observed by Michaelis and Rona (1908, 1909) and by Tachau.

When glucose solutions are added to fresh blood, so as to bring the total concentration of sugar up to about that of a severe case of diabetes, much of the added sugar is said to penetrate the corpuscles within two minutes (Rona and Döblin, 1911). It is also stated that repeated washing of the corpuscular sediment, by centrifuging with isotonic saline, causes the corpuscles to lose the power of absorbing glucose, which would indicate that some change has occurred, having the effect of reducing their permeability, or selective solubility, towards glucose. There is no evidence to justify the claim of Falta and Richter Quittner, that the corpuscles in living blood contain no sugar, but that this only diffuses into them after removal of the blood from the body.

Manner of Existence of the Sugar in the Blood.—The possibilities are (1) that all the sugar is in simple solution, as glucose; (2) that a part of it is in simple solution, the remainder being in combination, either loosely or firmly, with other substances, such as proteins; and (3) that part is in simple solution, the remainder existing as disaccharides or polysaccharides. There has been considerable speculation as to which of these possibilities actually represents the manner of occurrence of the sugar in the blood. Based on the fact that more sugar is often found after hydrolysing the blood with acid than before such hydrolysis, many observers have concluded that combined sugar occurs. To explain the well-known fact, that only small quantities of sugar exist in the urine so long as the blood sugar does not rise above what is known as the renal threshold for sugar, it has also been supposed that even the ordinary sugar of the blood is present in some form of loose combination. This imaginary sugar-compound is supposed to be of so loose a character that it immediately breaks down when the blood is treated with reagents for the removal of protein, prior to estimating the reducing power.

The only way by which the possibility of the existence of such a compound can be investigated by direct experiment is by observing, in fresh blood, the diffusibility of the sugar through membranes, it being assumed that the free, but not the combined sugar, will diffuse. Simple dialysis of blood against isotonic saline cannot be used for this purpose, for in such a case the sugar which diffuses through the dialyser from the blood into the isotonic saline will bring about such a lowering of the percentage of free sugar in the blood that the equilibrium, which must be assumed to exist between the free and the combined sugar,

will break down at a rate proportional to diffusion. As a matter of fact, when normal or diabetic blood is dialysed against a large amount of isotonic saline, all the sugar disappears in between twenty-four and forty-eight hours, even when the temperature is kept so low that no glycolysis can occur (see p. 194). When, on the other hand, the outside fluid is limited in amount, diffusion proceeds until the percentages of sugar within and without the dialyser are equal, a fact which has been clearly demonstrated in the so-called vivi-diffusion experiments of McGuigan and Ross (1917), and of J. J. Abel (1914).

An ingenious experiment in this connection is that of Michaelis and Rona (1908-09), in which equal quantities of fresh unclotted blood were placed in a series of small collodion dialysers, which were then immersed in isotonic saline solutions containing glucose in percentages which varied between 0·05 and 0·2 per cent. in the different solutions. After dialysing for twenty-four hours at low temperature, and with sterile precautions, analysis of the blood in the various dialysers showed that the percentage of sugar had not become changed in that one in which it had been dialysed against a solution containing the same percentage of glucose as the blood itself. In the other cases, the percentage of blood sugar was found either to have increased or decreased.

Although the results of these experiments would seem to indicate that all the sugar in the blood must be in simple solution, there are certain facts which are difficult to explain, unless it be assumed that some is also combined.

Kleiner, for example, found that during slow dialysis of diabetic blood (by using a thick membrane), the rate of diffusion of sugar (from hour to hour) was not uniform, although it was so with normal blood with glucose dissolved in it. He thinks that these results indicate that some of the sugar in diabetic blood exists in a combined state, and he assumes that this must also be the case for the sugar in normal blood.

Another fact that is difficult to explain is that the diffusion of sugar through the perfused kidney of the frog depends very largely on the saline composition of the perfusion fluid (Hamburger); for example, when this consisted of Ringer's solution containing less than 0·2-0·3 per cent. glucose, the fluid that escaped from the ureters contained only a little less glucose than that present in the perfusion fluid, whereas when the percentage of bicarbonate in the latter was raised (to 0·285 per cent.), so as to bring the reaction to that of normal frog's blood, the urine became sugar-free. Alteration in the concentration of the calcium of the perfusion fluid also had an effect on the sugar concentration of the urine. When other sugars, such as fructose or lactose, were added to the perfusion fluid they were passed quantitatively into the urine.

From time to time attention has been paid to the possibility that glucose may become united with the protein in blood, to form compounds that are broken up only by energetic chemical action. A review of the older work in this field was given by Pavy (in 1906) and by Langstein (in 1904), and some time later by Lépine (in 1909). Recently Grevenstuk and Laqueur, in their excellent monograph on "Insulin" have devoted considerable space to the subject, and Winter and Smith have pointed out the possible significance of such sugars in diabetes. There can be little doubt, as has already been remarked, that under certain conditions (changes in pH, etc.) protein, or rather amino acids, may form combinations with glucose, but it would appear that more work of a purely biochemical nature is required before the physiological significance of these compounds can be determined. My interest in these possible compounds was aroused anew during recent investigations on the blood sugar of fish, done in association with McCormick. We found that although hydrolysis of the protein-free filtrates did not, as a rule, greatly increase the reducing power, this became very marked when the laked blood was hydrolysed, prior to precipitation of the proteins. The following figures will serve to illustrate :—

(1) Mixed blood from five fish (*Myoxocephalus*) contained 0·024 per cent. glucose.

(2) Protein-free *filtrate* heated on boiling-water bath in presence of weak HCl contained 0·035 per cent. glucose.

(3) *Blood* heated in presence of weak HCl contained, after removal of the proteins, 0·075 per cent. glucose.

The masked sugar evidently exists in some colloidal form, so that it is precipitated by tungstic acid. It is considered possible that it is to a breakdown of such compounds that the blood sugar, as ordinarily determined, increases so rapidly during asphyxia in fish.

Bierry and his associates and Condorelli have devoted much attention to this subject.[1]

They point out that although the reducing power of blood becomes raised by hydrolysing it with acid at 120° C. in an autoclave, this does not occur significantly when protein-free filtrates are used (see also Folin and Berglund's results on p. 214). Neither glycogen nor glycuronates

[1] The papers by these workers not being at present available, the following account of their work is taken from Grevenstuk and Laqueur's article.

can be the source of this sugar, and since it is produced by similar hydrolysis of the blood proteins, but not by that of tissue proteins, it is believed to be a sugar compound peculiar to the blood. Whatever may be the chemical nature of the compound, the proportion which it bears to the free sugar of the blood is said to be a tolerably constant one, although this varies for animals of different species. The proportion is relatively small in birds, in which the free sugar is high, in dogs it amounts to about 50 per cent. of the total sugar, whereas in cold-blooded animals it is much higher. Bierry and Fandard call attention to the relationship between these differences and those in body temperature. They, as well as Condorelli, state that about 50 per cent. of the total sugar in the blood of man is bound, and whereas the free sugar is somewhat less in venous, than in arterial (finger) blood, the bound sugar remains unchanged, although it is thought to be less firmly united. After prolonged starvation, the bound sugar increases relatively as the free sugar falls, and after the injection of sugar, the bound sugar first of all diminishes (after twenty minutes), regains the normal level in about ninety minutes, and then rises above it. The curves of free and combined sugar, therefore, cross each other, the latter regaining the normal level after about two hours. Both in experimental (epinephrin) and in clinical diabetes, changes (usually a diminution) have been described in the amount of bound sugar, but the two groups of workers, mentioned above, are not agreed as to their extent. In any case these changes are probably of little significance, since similar ones have been described in other diseases, such as nephritis. Nitzescu has been able to confirm earlier work by Bierry and his school, that the bound sugar, after pancreatectomy, becomes increased, although relatively less so than the free. Both groups of workers, as well as Condorelli, have also described definite changes following the injection of insulin in normal and diabetic animals, and in diabetes mellitus. In general, the bound sugar increases, not, as a rule, at the same time as the decrease in free sugar, but decidedly later. The extent of the increase is not sufficient to account for all the sugar which disappears. Best and Scott have likewise found that acid hydrolysis of the blood of animals injected with insulin, or its partial digestion with trypsin, causes a decidedly greater increase in sugar than occurs when the blood of normal animals is similarly treated, although McCormick, Noble, and I had previously been unable, in two experiments on insulin-treated rabbits, to obtain results that were different from those on normal ones. In any case, it is evident that the formation of this bound sugar cannot be a significant factor in accounting for the sugar which disappears, especially when this becomes extensive, as during the injection of sugar at the same time as insulin (p. 300).

Reference may be made here to the work of Forrest, Winter, and Smith, in which they compared the reducing, with the polarising power of the blood sugar in normal and diabetic persons.

In the former the dextro-rotatory power was relatively less than the reducing, from which, as we have seen, they concluded that γ-glucose must be present, whereas in diabetic blood the reverse was the case, indicating the presence of polysaccharides. By mild hydrolysis the isolated sugar of diabetic blood became still more dextro-rotary without any change in reducing power, but by stronger hydrolysis both increased, so that after some time polarising and reducing powers came to correspond to those of an equilibrated mixture of α-, β-glucose. By treatment with insulin, five out of six cases of diabetes showed, after two to three days, a return of the polariscopic and reduction values to those of normal persons. As is shown on the table on page 186, Eadie has also observed a deviation from the normal relationship between polarisation and reduction values in the blood of animals injected with epinephrin (a fact also observed by Winter and Smith), but it is scarcely safe at present to speculate as to the significance of the observation.

Lépine's statement that an increase in free sugar occurs at body temperature in blood immediately after it has been removed from the body cannot be confirmed.

For example, when blood is collected from the blood-vessels in ice-cold water and immediately precipitated with colloidal iron it has always shown, in my experience, a greater concentration of glucose than in other portions of the same blood simultaneously withdrawn into flasks and incubated for varying periods at body temperature (Macleod, 1913). Glycolysis would seem to set in immediately blood is withdrawn without there being any preliminary production of free sugar.

Lépine also believed he could detect a higher concentration of glucose in blood removed from the carotid artery than in that simultaneously removed from the inferior vena cava just above the liver. He explained this as being due to hydrolysis of non-reducing carbohydrates occurring in the blood during its passage through the lungs. If this result could be confirmed, which it has not been, it would indicate that some of the carbohydrate which is discharged into the blood by the liver appears not as glucose, but as products which are intermediary between glycogen and glucose; lower dextrines perhaps. It is of interest in this connection that Huber and I (1917) have been able to confirm the observations of Ishimori, working with Hofmeister,

that material giving the carmine stain for glycogen can be observed under the microscope to be extruded from the liver cells into the hepatic capillaries in conditions in which there is increased glycogenolysis, such as can be brought about by piqûre or by stimulation of the great splanchnic nerves. I have attempted to identify, by chemical means, such condensation products of sugar in protein-free filtrates of the hepatic blood under similar conditions, but without success.

Taking these observations as a whole, it may be said that there is, at present, no evidence to contradict the view that all of the immediately available sugar in the blood exists there in simple solution, partly in the plasma and partly in the corpuscles.

Glycolysis in Blood.—After the blood has been drawn from the body sugar disappears from it at a rate which varies with the temperature at which it is kept, and with the species of the animal. In normal dog blood, kept at body temperature, about 50 per cent. of the sugar disappears in about two and a half hours. In that of the pig, sheep, and rabbit, on the other hand, much less disappears in this time. Not only does the rate of glycolysis vary in the blood of different species, but also in that of individuals of the same species, and even in that of the same individual at different times. This has led some to suggest that the rate of glycolysis may bear a relationship to carbohydrate metabolism, but there is no certain evidence that this is the case. Thus, glycolysis cannot be shown to be effected by diet, and it is, as a rule, more marked in the blood of the carnivora than in that of the herbivora.

Lépine (1909) and others have stated that glycolysis becomes depressed in the blood of diabetic patients, and that its behaviour in this regard runs parallel with diminution in the sugar-oxidising power of the tissues, but few agree with this view. Indeed, it is highly improbable that the rate of disappearance of sugar from the blood bears any relationship whatsoever to the rate of its metabolism in the organism. Thus, Pearce and I (1913) found that sugar disappears from the blood of animals from which the liver had been removed at a rate which was many times greater than that at which it disappeared from the blood of these animals kept at body temperature outside the body. In eviscerated dogs, for example, from 0·83-4·46 mgs. glucose disappeared from 100 gms. of blood per minute, whereas only 0·03-0·06 mg.

disappeared from the same blood *in vitro*. More recently, Denis and Giles have investigated the relationship between glycolysis and diabetes, with results which seem to indicate that in severe cases glycolysis is much less than normal, being practically absent in coma.

The condition in which the blood is kept after its removal from the body may influence the rate of glycolysis considerably. For example, potassium oxalate in concentrations of 0·1 per cent. or over retard it, and fluoride inhibits it almost entirely. It is not affected, however, by hirudin. Agitation of the blood accelerates the process, in comparison with unagitated blood, and this difference appears to depend on the amount of free oxygen, since the process is decidedly aided by bubbling oxygen slowly through the blood. The process still goes on, however, in the absence of oxygen. Glycolysis does not occur in serum or plasma free of leucocytes, but occurs very rapidly in the centrifuge deposit of blood. Both hæmocytes and leucocytes probably participate in it. Suspension of leucocytes, such as sterile pus diluted with Locke's solution, have a high glycolytic power, and Levine and Meyer (1912) have used this as a means of studying the process. Hæmocytes are also said to be able to show it in the absence of leucocytes (Rona and Arnheim, 1913). On the other hand, frequent washing of hæmocytes by centrifuging with isotonic saline, robs them of their glycolytic power. This is of interest because it has been found that such treatment also renders the corpuscles incapable of absorbing glucose, which would suggest that glycolysis is an intracorpuscular process. There seems to be a relationship in the blood of different animals between the rate of glycolysis and the permeability of the red blood corpuscles for glucose. Thus, glycolysis is poorly developed in the blood of the rabbit, the corpuscles of which are relatively impermeable to glucose, whereas, on the other hand, it is rapid in the blood of the dog, in which, as we have seen, the corpuscular permeability is high. Glycolysis does not occur in fish blood (McCormick and Macleod).

The reducing sugar, which disappears during glycolysis, may either become broken down into non-reducing sugar derivatives, or polymerised to form disaccharides or lower dextrines. We have already seen that the reducing power becomes increased when blood is hydrolysed, by heating with acid. Levine and

Meyer found that this increase is proportionally greater in blood after glycolysis, which would seem to indicate that the sugar which disappears has become polymerised. It has been pointed out, however, that considerable quantities of sugar were added to the blood to start with in these experiments, and we are not aware that anyone has shown that in normal blood similar results are obtained. The same authors, using suspensions of leucocytes, found that lactic acid accumulates in the blood as the sugar disappears. Mellanby has also found that lactic acid accumulates in blood after removal from the body, and he accounts in this way for the diminution in the alkaline reserve which also occurs.

Since it is unlikely that the glycolysis occurring in shed blood corresponds in any way to the utilisation of the sugar in the intact animal, a study of the process can be of very little value in the investigation of the problem of carbohydrate metabolism.

Concentration of Sugar in the Blood of Man and of the Lower Animals.—In comparing the blood sugar concentration in the blood of different animals, it is essential to bear in mind that a definite increase occurs shortly after the ingestion of carbohydrate-rich food. It is necessary, therefore, to remove the blood some time after the last meal, and in man the practice is to do this the first thing in the morning before food has been taken. Immediately after its removal the blood must be precipitated, so as to prevent glycolysis. In man it is usually the venous blood that is observed, because of the difficulty of removing arterial blood. An approximation to the latter may be obtained by taking blood from a prick at the base of the finger nail. The difference in percentage of sugar between arterial and venous blood is, however, tolerably constant, so that for most purposes venous blood can be employed without much danger of serious error (Henriques and Ege, 1921).

The table on the opposite page gives the extent of variation which may be expected in the blood of man.

It will be observed that the lowest value is 0·045 per cent. and the highest 0·12 per cent. There is reason to suspect, however, that this low value may be due to experimental error, since it is now known, as the result of investigations with insulin, that when the blood sugar of man falls to 0·07 per cent. definite symptoms of hypoglycæmia supervene. In forming this opinion

Number of Cases Observed.	Variations in Blood Sugar.	Method Used.	Observers.
30 adults	0·05-0·12	Original Lewis-Benedict	Gettler and Baker.
,,	0·06-0·11	Bang micro	Hopkins.
500 ,,	0·09-0·11	Modified Lewis-Benedict	Myers and Bailey.
16 ,,	0·09-0·12 0·085-0·11	Modified Lewis-Benedict	Denis, Aub, and Minot.
113 ,,	0·07-0·14*	Original Lewis-Benedict	Williams and Humphreys.
,,	0·09-0·11		Lewis and Benedict.
26 children	0·072-0·113	Original Lewis-Benedict	Bass.
100 ,,	0·087-0·118	Bang micro	Goetz.

* Some cases were examined one hour after taking food.

it must be borne in mind, however, that these symptoms may be due to the rapidity in the fall of blood sugar, rather than its absolute level, for it has been observed by Dr. Wagner of Vienna that blood sugar considerably lower than this can occur in clinical cases without any symptoms.

In the blood of lower animals the sugar concentration is in general of the same magnitude as in the blood of man, as is illustrated in Table XVIII.

It is interesting to observe that even in the invertebrates the concentration of sugar, though decidedly lower, as a rule, sometimes approaches that for mammalian blood. In the circulating fluids of the molluscs and of octopus it is particularly low.

With regard to the low percentages sometimes found in the dogfish (*Squalus acanthias*), it may be stated that some observers, Diamare, for example, have been unable to find any trace of sugar, and it is the case that in many specimens of this animal observed by us no reducing substance could be detected. On the other hand, E. L. Scott (1921) found that under certain conditions there might be about as much sugar in the blood of the closely related *Mustelus canis* as in mammalian blood. These workers also found that the blood sugar fell to a trace in specimens of this fish that were in a moribund state.

Although it is not certain that the sugar in the blood of the lower animals is chemically identical with that of the mammals, it may be pointed out that it must be closely related, since it forms typical crystals of glucosazone with phenyl hydrazine. It remains to be seen, however, whether it is fermentable by yeast, whether it gives the other biochemical reactions of glucose, and whether it rotates the plane of polarised light to a similar degree. If, as seems probable, glucose should prove to be

TABLE XVIII.

Animal.	Per Cent. Reducing Power.			Observer.
	Maximum.	Minimum.	Average.	
Dog	0·102	0·073	0·085	Oppler and Rona.
Dog immediately after etherisation)	0·146	0·075	0·111	Macleod and Pearce.
Cat	0·096	0·056	0·069	E. L. Scott.
Rabbit	0·13	0·08	0·10	Oppler; Bang.
Sheep	—	—	0·07	Bodansky.
Marmot—				
1. Hibernating	—	—	0·009	Dubois (cf. Bierry and Fandard).
2. Active	—	—	0·117	———
Birds—				
Chicken	—	—	0·23	Bierry and Fandard.
Duck	0·188	0·117	0·016	Weintraud
Geese	0·160	0·120	—	Kausch
Fowl	0·250	0·188	0·209	Saito and Katsuyama
Pigeon	0·430	0·160	0·185*	Scott and Honeywell
Fowl	0·255	0·235	0·245	J. Markowitz.
,,	0·254	0·173	0·211	,,
Tortoise	0·21 †	0·05	0·102	Unpublished.
Frog (Rana temperaria and esculenta)	0·05	0·02	—	Bang.
Normal frogs in July	—	—	0·035	Lesser.
,, ,,	0·065	0·040	0·053	Brinkman and van Dam.
Rana pipiens—				
(February-April)	0·053	0·011	0·037	E. L. Scott.
Males	—	—	0·033	———
Females	—	—	0·040	———
Fish: Teleostomi—				
Carp (Cyprinus)	0·145	0·058	0·090	Lang and Macleod.
Sculpin (Myoxocephalus)	0·061	0·007	0·030	McCormick and Macleod.
Haddock (Melanogrammus)	0·083	0·028	0·055	,, ,,
Cod (Gadus)	0·070	0·061	0·065	,, ,,
Sea raven (Hemitripterus)	0·186	0·08	0·082	,, ,,
Brook trout	—	—	0·100	Macleod and Noble.
Chimæra (rat fish)‡	0·037	0·022	0·028	Lang and Macleod.
Elasmobranchus (dog fish) Squalus	0·038	0·000	Trace.	,, ,,
Mustelus canis	249 §	0 ‖	0·065	E. L. Scott.
Carcharias littoralis (sand shark)	0·077	0·027	—	,,
Raja (skate)	0·068	0·018	0·038	McCormick and Macleod.
Molluscs (horse clam)	—	—	Trace (?)	Lang and Macleod.
Arthropoids—				
Cancer productus	0·081	0·039	0·040	,, ,,
,, irrotatus	0·11	0·06	0·074	,, ,,
Octopus	—	—	0·032	Bierry and Giaja.

* Excluding three very high results. † Possibly asphyxiated. ‡ Fish dead some time before blood removed. § Asphyxiated. ‖ Practically dead.

practically the only sugar present in the blood of all animals, the fact would be of great interest when we consider that many, such as certain molluscs, live on carbohydrate foodstuffs which are composed mainly of pentosans, by the hydrolysis of which, not hexose, but pentose sugars result. Since, however, in these animals glycogen, giving glucose on hydrolysis, is deposited in the tissues, it is altogether likely that the blood sugar is also glucose.

Special interest attaches to the blood sugar in the fishes, and for various reasons, among which the following may be mentioned :—

(1) These animals live on food with very little preformed carbohydrate, so that gluconeogenesis must be constantly going on.

(2) The blood sugar of deep-sea fish of different species living in temperate zones is remarkably constant (from about 0·020-0·050 per cent.), provided the blood is collected immediately after catching (McCormick and Macleod).

(3) The slightest interference with respiration, as by a short exposure to air, causes hyperglycæmia.

(4) Hydrolysis of the blood by heating with weak acid causes an increase in sugar, which is very pronounced as compared with that occurring in mammalian blood (p. 191).

(5) In many of the Teleostei it is possible to induce hyperglycæmia by removing the islet tissue (principal islets) without disturbing the pancreas (p. 35).

The relatively low blood sugar of deep-sea fish contrasts with the much higher values obtained in river fish, such as the trout. There is no evidence to indicate that this difference depends on the continual muscular activity of the latter, as compared with the quiet swimming of the former fish.

The Blood Sugar in Laboratory Animals.—Because of its importance for experimental purposes, the blood sugar in laboratory animals has been very carefully determined by various workers, especially in the rabbit, the cat, and the dog. Since in all these animals a certain degree of variation is observed, even when the conditions of feeding, of housing, and of preliminary treatment are standardised as far as possible, it is necessary to know, not only the average, but also the extent of deviation. Using reliable modern methods of blood sugar estimation, the following results may be taken as standard :—

The Rabbit.—E. L. Scott and T. H. Ford (1922), and G. S. Eadie (1922) simultaneously determined the values in this animal, both groups of workers treating them statistically by the usual methods, with the following results :—

Observer.	No. of Animals.	No. of Obs.	Mean.	Median.	Standard Deviation.	Probable Error.
Scott and Ford .	27	85	118	116	21·5	—
Eadie .	—	157	116	115	12·2	8·2

In Scott's and Ford's observations, therefore, the maximum blood sugar was 138 and the minimum 98, the corresponding figures in Eadie's, being No. 127 and 103.[1]

The observed animals had not fed for twenty-four hours before the observations, and only one specimen of blood was removed from each (in the morning). Both groups of workers also determined the sugar in blood removed at regular intervals throughout the day, Eadie finding in a large number of rabbits that a definite fall occurs between 1 and 3 P.M. The mean values (mgs. per cent.) for a considerable number were as follows :—

9-10 A.M., 118 per cent. ; 10-11 A.M., 118 per cent. ; 11-12 NOON, 120 per cent. ; 12-1 P.M., 115 per cent. ; 1-2 P.M., 112 per cent. ; 2-3 P.M., 112 per cent. ; 3-4 P.M., 114 per cent. ; 4-5 P.M., 117 per cent. Whether this slight but undoubted drop in the afternoon hours is of any physiological significance, we cannot say.

The Dog.—The observations on this animal are not nearly so numerous, if we omit those made under anæsthesia. In blood collected by needle from the saphenous vein, or from the heart, of animals accustomed to the operation, and in which, therefore, no element of excitement entered in, the following percentage results have been obtained :—

0·096, 0·092, 0·074, 0·092, 0·088, 0·096, 0·093, 0·085, 0·092. With the Folin-Wu method, Morgulis found between 0·074 and 0·106 per cent. in five dogs.

[1] The formula used was $\alpha = \frac{\sqrt{\Sigma d^2}}{n-1}$,
where α = standard deviation,
d = the individual variations of each value from the mean,
n = the number of observations.
The probable error equals 0·6745 α.

Shaffer (1914) examined blood taken from the jugular vein without anæsthetic in five dogs, and gives the average sugar concentration as 0·05 per cent., which is about one-half that usually accepted as the normal. The method used in these observations has since been superseded by another devised by the same author in association with Hartmann, but we are not aware of observations made by its use on the blood sugar of normal dogs. Embden, Lüthje, and Liefmann (1907) also give low values for normal unanæsthetised dog, viz. 0·057-0·088 per cent.

The Cat.—A very careful investigation of the blood sugar in this animal was made by E. L. Scott (1914) at a date (1913) prior to the introduction of micro methods. It was therefore necessary to obtain large amounts of blood, for which purpose the animals were decapitated, the method of analysis being a modification of that originally described by Waymouth Reid. There were twenty-two observations, and the mean value found was 0·069 per cent., the maximum being 0·096 and the minimum 0·056. The same author quotes results by Pavy, in which the blood was taken from the heart after pithing the animal, as follows: Mean, 0·088; maximum, 0·103; minimum, 0·068.

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CHAPTER XIV.

EXOGENOUS HYPERGLYCÆMIA AND GLYCOSURIA.

Although, as we have seen, the percentage of sugar in the blood, measured in the post-absorptive condition, is remarkably constant and does not vary greatly among animals of different species, it may be caused to rise far beyond the normal level by a variety of experimental conditions, apart from those which cause diabetes. These various forms of experimental hyperglycæmia are usually considered to have in common one feature which distinguishes them from diabetes, and this depends on the fact that the extra sugar comes from an excess which is either absorbed from the intestine or liberated from the glycogen stores of the liver, so that when absorption is completed, or the stores of glycogen have become exhausted, the blood sugar returns to the normal level. We shall see, however, that this distinction is not without exceptions, there being certain forms of experimental hyperglycæmia, such as that due to epinephrin, which persist after all glycogen has disappeared from the liver. As a secondary effect of the hyperglycæmia, there is usually glycosuria, but sometimes, as when the excretion of urine is curtailed by a lowering of the blood pressure, this symptom may fail, or at best be slight in comparison with the hyperglycæmia. It is important to hold this fact in mind, since not a little confusion in the results of different workers can be attributed to a failure to do so.

For convenience, the conditions causing hyperglycæmia may be divided into two main groups, Exogenous [1] and Endogenous. In the present chapter we shall discuss the main features of the exogenous forms, and the effect of insulin on them. At the

[1] This term is adopted rather than "alimentary" or "post prandial," since the latter cannot be used when the sugar is added by subcutaneous or intravenous injection.

same time it will be convenient to consider the relationship between the level of sugar in the blood and the appearance of sugar in the urine, the laws governing this relationship being presumably the same for all forms of experimental diabetes.

Exogenous Hyperglycæmia and Glycosuria.

1. In Laboratory Animals.—In 1913 Bang and his pupils showed that the blood sugar of the rabbit rose in about fifteen minutes after the administration, by stomach tube, of from 2 to 10 gms. of glucose, the extent and duration of the rise varying with the quantity of sugar ingested. Hyperglycæmia developed also with starch when this was given to starving animals, but when it was given to rabbits in which digestion was already in progress, no change in blood sugar was found to occur. The interpretation put upon these results was that the liver does not succeed in retaining as glycogen all of the glucose that is absorbed from the intestine into the blood of the portal circulation, at least when absorption is rapid.

E. L. Scott and Ford (1922) have more recently extended these observations with the object of determining the degree of variability to be expected when different animals are similarly injected. They gave from 1 to 4 gms. of glucose per kilo body weight, by mouth, to animals previously fasted for twenty-four hours, with the following results: 1 gm. caused the blood sugar to rise to a maximum of 175 mgs. in thirty minutes, the original level being regained in three hours; with 2 gms. the maximum reached was 190 mgs. in about forty-five minutes, and the blood sugar was still decidedly (8 per cent.) above the original level in three hours; with 4 gms. the maximum reached was 235 mgs. in one hour, and there was still 20 per cent. excess of sugar in three hours. There was, however, great variability in the extent to which different animals reacted. A significant fact, emphasised by Scott and Ford, is that the extent of the hyperglycæmia after thirty minutes was the same with the 2 gm. as with the 4 gm. doses, the only difference being that the blood sugar after this time continued to rise with the larger dose, whereas it began to fall with the smaller one. Since the curves were of equal height up to thirty minutes, the conclusion was drawn that the maximum rate of absorption must be

attained when 2 gms. of glucose are ingested, which corresponds closely with the figure arrived at by Woodyatt (1·8 gm. per kilo), by the method of continuous injection (see p. 218). Following the hyperglycæmia, with the smaller doses of glucose, a decided degree of hypoglycæmia was observed to occur. This confirms the observations of Jacobsen (1913), Maclean and de Wesselow (1921), and Folin and Berglund (p. 213), made on man. The hypoglycæmia probably depends on stimulation of the internal secretion of insulin by the hyperglycæmia.

Eadie's observations differed from the foregoing in that the sugar was given subcutaneously, the doses being 0·8–1 gm., and 1·6–2·0 gms. per kilo body weight. With 1 gm. and 2 gms. results similar to those of Scott and Ford were obtained, except that the curves descended more slowly, and in no case was any hypoglycæmia found to follow. This may depend on the method of administration, and may signify that stimulation of the internal secretion of insulin occurs only when sugar is absorbed directly into the portal blood. In both groups of observations great deviations occurred in the extent of the hyperglycæmia resulting from the injection of equal quantities of sugar into different animals, which indicates the unsuitability of using injected animals for the assay of insulin. In the majority of Eadie's experiments a secondary rise in blood sugar was observed to occur after the initial level had been reached. His results are depicted in the curve of Fig. 17, in which the vertical lines indicate the standard deviation (see p. 200) for the different injections.

Insulin has a striking influence on exogenous hyperglycæmia. This is shown in the chart of Fig. 18, in which the degree of hyperglycæmia is compared in a series of rabbits, all injected subcutaneously with the same quantities of glucose, with or without insulin. Each of the vertical lines represents a period of three hours, and the horizontal ones indicate the degree of hyperglycæmia attained. The first curve shows the average for normal rabbits and each of the others, the effects produced when the sugar was injected at the same time as insulin (No. 1), or at varying periods (indicated by the times on the abscissa) after it. It will be seen that when insulin and sugar are injected together, both the extent and the duration of the hyperglycæmia are strikingly reduced, but the most pronounced effect is obtained

when the sugar is injected in from seventy-five to ninety minutes after the insulin. After this interval the influence of insulin wears off, so that in about four hours the sugar causes nearly

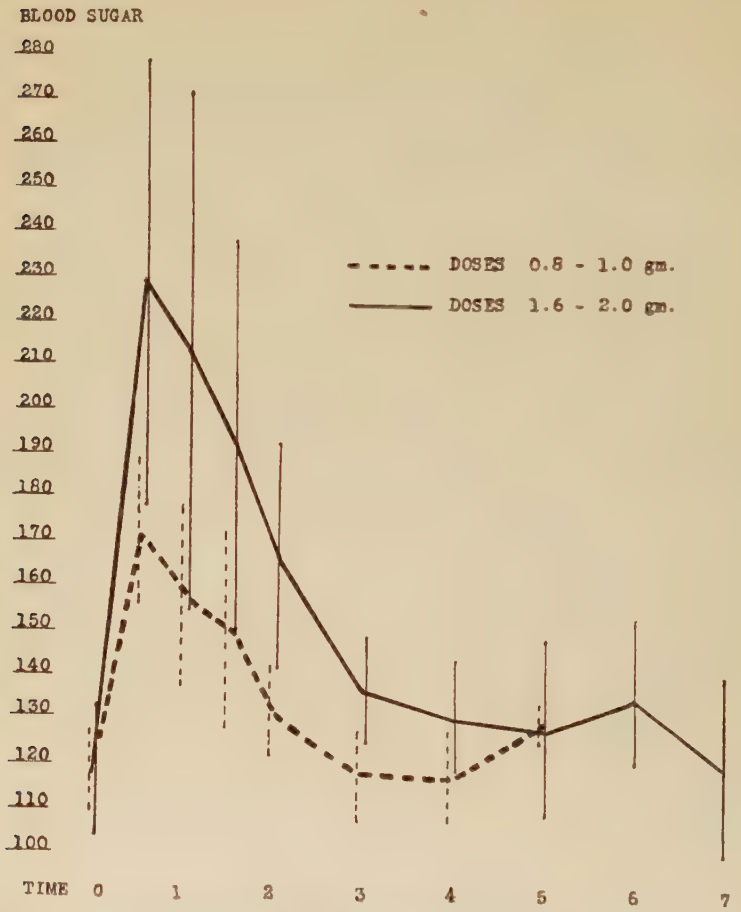

FIG. 17.—Curves showing average rise in blood sugar following the administration to rabbits (subcutaneous injection) of the quantities of sugar indicated. The vertical lines show the standard deviation for the different injections. (Eadie.)

as much increase in blood sugar as when it is injected into normal animals.

2. In Man.—Stimulated by Bang's observations on rabbits, Jacobsen (1913) undertook an investigation of a similar type on human subjects, in order to test the possible value of the method

for the diagnosis of diabetes. He examined, by Bang's micro-method, the sugar contained in blood collected from the finger, and he also examined the urine for the presence of sugar, using the ordinary clinical tests available at that time. The blood sugar determinations were made every fifteen minutes after giving 100 gms. glucose by mouth, sometimes before, and sometimes two or three hours after breakfast. He had two problems in view in making these observations: (1) To determine the contour of the blood sugar curve following the ingestion of this amount of sugar; and (2) to find out at what percentage

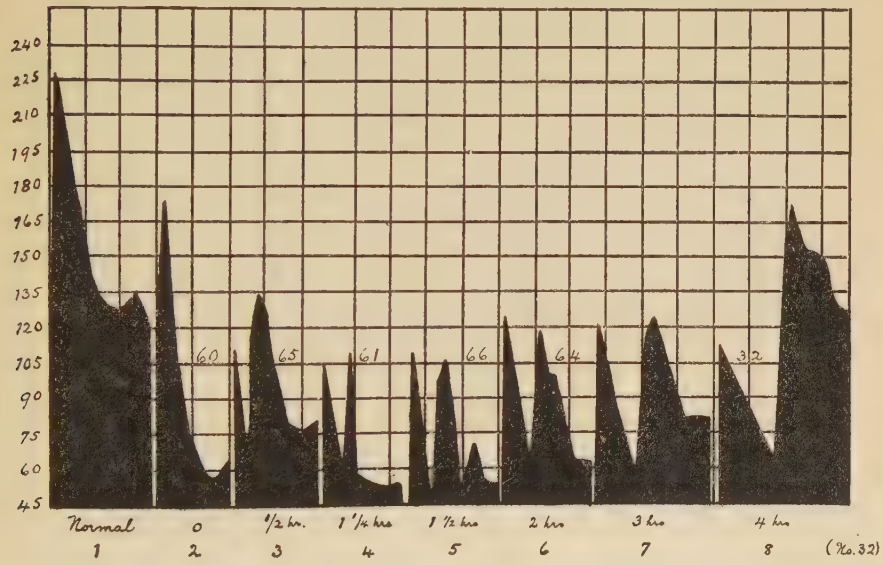

FIG. 18.—Chart showing influence of insulin on exogenous hyperglycæmia.

of sugar in the blood, quantities detectable by the usual sugar tests would appear in the urine, this being designated as *the renal threshold for sugar*.

In observations on fourteen subjects, Jacobsen found the blood sugar to rise to 0·170 per cent., or higher, in nine cases, and in all of these the urine gave a positive test for sugar. In four other cases the rise was only to 0·12 and 0·13 per cent. It must be borne in mind that the blood in these observations was received by pricking the skin at the base of the finger nail, so that it was mainly arterial blood, in which, presumably, blood sugar would be higher than in venous (because of the retention of sugar by the muscles, see p. 303). There is also a

possibility, as suggested by Folin and Berglund (1922), that the pain caused by the repeated needle pricks might have had an influence on the blood sugar. Since Jacobsen's contributions, many others of a similar nature have appeared. Thus, Hopkins (1915), in observations on eight persons, found the blood sugar to rise, as the result of 100 gms. glucose, to between 0·11 and 0·146 per cent., and in six, examined by Hamman and Hirschman (1917), it rose to between 0·1 and 0·14, following the ingestion of from 100 to 200 gms. of glucose. In none of these observations did sugar appear in the urine. Hagedorn (1921) (quoted by Folin and Berglund) observed alimentary glycosuria after giving from 36 to 120 gms. of glucose before breakfast. In the light of these and many other investigations of a similar nature, the renal threshold for glucose is considered to stand at 0·16 to 0·18 per cent.

With regard to the *time* at which blood sugar reaches its maximum, and the *duration* of the hyperglycæmia, following sugar ingestion in man, there is considerable irregularity.

In Hamman and Hirschman's observations the maximum was reached in from twenty to ninety minutes, and the duration of the hyperglycæmia varied from forty-five minutes to three-and-a-half hours. In Jacobsen's observations, however, the maximum was reached in about half an hour in those cases in which it stood at a low level, but might be delayed for ninety minutes in cases in which the maximum was high. In Hopkins' observations the maximum was reached in about thirty minutes in half of the eight cases examined, but both the height to which the blood sugar rose and the duration varied greatly in the other four cases. It will be evident from these data that no hard and fast rules can be laid down regarding either the extent or the duration of the post-prandial sugar curve. Certain clinicians, such as Maclean, Henry J. John, and Williams and Humphreys, put considerable value on the study of such curves in the diagnosis of early diabetes. When it is used for this purpose the sugar (100 gms. glucose) should be given in the post-absorptive state and the blood sugar examined at least at half hourly intervals. Excellent accounts of the clinical aspects of this work have been given by Hamman and Hirschman (1917), Bailey (1919), Williams and Humphrey (1919), John (1922), Maclean (1922), and G. Graham (1923).

The chief value in determining the renal threshold for sugar is in connection with the diagnosis of *renal glycosuria*, as contrasted with true diabetes. In the former condition, which is regarded as more or less benign, glycosuria occurs at levels of blood sugar distinctly below the threshold. In determining these relationships, however, there are several difficulties, and the reasons for these are as follows: (1) It is almost impossible to make certain that the blood sugar observed is actually at the

highest level attained during the period following the ingestion of the sugar. It may happen that before, or after the removal of the blood for analysis, the blood in circulation in the body, for a short period at least, contains considerably more sugar; (2) even when the blood and urine are removed as close together as possible they cannot be considered as strictly simultaneous samples; (3) while the blood sugar curve is descending the relationship between the sugar concentration in the blood and the sugar of the urine may be quite different from what it is while the curve is ascending (p. 217); and (4) the blood that is examined is usually venous blood, and since it is known, as the result of various investigations (Henriques and Ege, 1921, Macleod and Fulk, 1917, etc. (see p. 303)), that the venous blood of the muscles contains distinctly less sugar than the arterial, it is clear that there may be considerably more sugar in the arterial blood, at certain stages following sugar ingestion, than is revealed by the examination of the venous blood. Another factor which must influence the results is the rate of urine formation, but we know of no data from which its significance can be clearly worked out.

These remarks will serve to show how difficult is the precise determination of the renal threshold. We cannot here review all of the work in this field, but reference may be made to that of Goto and Kuno (1921).

The observations were made on fifty-three Japanese subjects, and in them plasma was used for the sugar determinations; twenty-two of the subjects showed sugar in the urine following the ingestion of 100 gms. glucose, dissolved in 250 c.c. of water, taken first thing in the morning. In eight of these the sugar threshold was between 0·180 and 0·190 per cent.; in five, between 0·160 and 0·172; and in eight, between 0·128 and 0·155 per cent. The sugar having once appeared in the urine, as the result of hyperglycæmia, remained in it long after the blood sugar has descended below the level at which the glycosuria set in. This fact had previously been emphasised by Bailey; and more recently, by Folin and Berglund. Indeed, sometimes, following the ingestion of sugar, the glycosuria remains even after the blood sugar curve has descended to a lower level than the normal for that individual. Although the upper limit of the sugar threshold can, therefore, be determined with tolerable accuracy at 0·170 per cent., the lower level can be much less definitely placed. In some cases, indeed, as pointed out by Faber and Norgaard (cf. Folin and Berglund), there may be a transient mild glycosuria after every meal. These facts are of evident

importance in connection with the diagnosis of renal glycosuria, in which the sugar threshold of the kidneys stands at so low a level that it is difficult to make the urine sugar-free.

In the foregoing observations the test used for sugar in the urine is given only when the concentration reaches a certain level. On the other hand, there are known to be present in this fluid quantities of reducing bodies closely related to sugars, but which can be detected only after removing from the urine substances which interfere with the reduction reaction. The notion of the renal threshold presupposes that until a certain level has been reached by the sugar in the blood none of this will overflow into the urine, this hypothesis being that originally propounded by Claude Bernard. Demonstration of traces of sugar, or related carbohydrates, in normal urine, however, has made it necessary to reconsider this theory, and this has been done, particularly by S. R. Benedict and Osterberg and by Folin and Berglund. The former investigators deny the significance of a renal threshold, whereas the latter place great importance on it. We will review these important researches in some detail.

Benedict, Osterberg, and Neuwirth (1918) used mercuric nitrate to remove the interfering substances (urochrome, uric acid, creatinine, etc.) from the urine, and they determined both fermentable and non-fermentable reducing substances in the filtrates.

The observations were made on two normal men, of fifty-five and twenty-two years of age respectively. On ordinary diets the total daily excretion of reducing substance varied between 0·7 and 1·16 gms., rising to 1·6 when the food contained excess of carbohydrate. These variations did not immediately follow the changes in the carbohydrate content of the diet—as is the case in diabetes—but became evident after several days. The figures correspond fairly closely with those previously obtained by Macleod, Christie, and Donaldson (1912), who removed the interfering substances by means of charcoal in the presence of 25 per cent. glacial acetic acid, and who found in the twenty-four hour urine of two normal men, living on mixed diets containing considerable amounts of carbohydrate, quantities of reducing substance varying from 1 to 1·85 gms.

Benedict and Osterberg also examined the urine voided at two-hour intervals during the day, so as to be able to correlate changes in the reducing substances with the ingestion of food. Following each meal, an increase was observed, particularly in the fermentable reducing substances, accompanied, sometimes, by a decided decrease in the non-

fermentable moiety, but the extent of the increase was not always proportional to the carbohydrate content of the diet. Occasionally, especially in the older man, the post-prandial increase in fermentable sugar became so marked that the urine gave a positive reaction with the ordinary clinical tests for sugar. Similar observations were also made following the ingestion of weighed quantities of glucose. Even 20 gms., although it did not alter the total of reducing substances, caused the non-fermentable moiety to become absolutely and relatively diminished. With 40 gms. a slight increase was observed in the total sugar excretion, accompanied again by a decided decrease in the non-fermentable moiety. When sugar was given following a previous administration of this substance, its influence upon the urinary excretion was less marked, indicating that the first ingestion had altered the assimilative powers of the organism. Somewhat similar results were obtained when, instead of giving the glucose on an empty stomach, it was given along with an average meal. In this case, however, the elimination of fermentable sugar was decidedly greater, and the non-fermentable sugar diminished in amount. All of these results were more marked on the older subject and somewhat similar ones were observed in the case of two dogs kept in metabolism cages.

The authors consider that their results indicate that the doctrine of a definite renal threshold should be abandoned. They say that " the normal organism is truly diabetic in that it has no absolute tolerance for carbohydrate." If this be true, then it is evident that the term " glycosuria " signifies nothing more than the presence in the urine of a sufficient concentration of sugar to give the ordinary qualitative tests, and Benedict suggests that, in place of this term, we should use that of " glycuresis " to indicate the condition in which the sugar normally present in the urine becomes increased in amount. He points out that if the total urinary sugar on an ordinary diet exceeds 1·5 gm. in twenty-four hours, the case should be further investigated, with the possibility in view that the assimilative powers for carbohydrate may be below the normal.

The investigations of Folin and Berglund differ from the foregoing essentially in the fact that blood sugar was measured at the same time as the elimination of sugar in the urine. The sugar was determined before and after hydrolysis, both in blood and urine, so as to give information as to how much of it could be considered as glucose. Both plasma and blood were examined, careful attention being given, when the former was used, to the quick separation of the plasma from blood, the clotting of which was delayed by coating the centrifuge tubes with paraffin, so as

to avoid using anti-coagulants. The results of these investigations were briefly as follows :—

Following the ingestion of pure glucose during fasting, the level of the blood sugar rose, but not sufficiently so as to overstep the renal threshold, provided there was entire absence of emotional conditions, and at no time during the increase in blood sugar was there any increase in the sugar excreted per hour by the urine. A typical experiment is shown in Table XIX.

TABLE XIX.

Subject, D—n. Age, 22 years. Weight, 75 kilos. 200 gms. glucose taken.
Result: Maximum sub-threshold hyperglycæmia but no glycuresis.

Time: Feb. 25, 1921.	Blood. Sugar per 100 c.c. Whole Blood.	Plasma.	Corpuscles.	Corpuscles.	Urine. Volume per Hour.	Sugar per Hour.	Remarks. Fasting.
A.M.	mg.	mg.	mg.	vol. per cent.	c.c.	mg.	
10.12	—	—	—	—	—	—	250 c.c. water taken. Urine voided.
11.55	105 + 0	107 − 9	102 + 12	43	—	—	———
P.M.							
12.00	—	—	—	—	50	21 + 3	———
12.05	—	—	—	—	—	—	200 gms. glucose taken in 830 c.c. water.
12.50	152 + 4	172 − 4	122 + 17	41	—	—	———
1.30	—	—	—	—	111	18 + 2	———
1.50	121 − 6	127 − 13	112 + 4	41	—	—	———
2.30	—	—	—	—	35	23 + 4	———
3.00	136 − 21	143 − 13	127 − 34	41	—	—	———
4.00	—	—	—	—	29	23 + 5	———
4.35	105 − 3	105 − 7	105 + 2	42	—	—	———
5.30	—	—	—	—	27	21 + 2	———
6.05	95 + 3	101 − 4	88 + 9	—	—	—	———
6.50	—	—	—	—	76	22 + 4	———
7.00	—	—	—	—	—	—	Dinner.
9.25	—	—	—	—	111	65 + 21	Note glycuresis after meal.

Small, plus or minus, figures show increase or decrease after hydrolysis of the protein-free filtrates.

On the other hand, in this, as in all the other five experiments of a similar type that are reported, a decided increase occurred in the sugar of the urine in the hours following the taking of a mixed meal (dinner). It would appear, therefore, that the sugar of normal urine is quite independent of the blood sugar. To quote the authors: "Hyperglycæmia below the threshold does not normally produce the slightest leaking of glucose through the kidneys, and normally not a trace of circulating glucose is lost." The effect of hydrolysis was variable and

of significance only in the case of the sugar of the urine, in which it indicated the presense of di- or poly-saccharides.

With fructose, galactose and lactose, as also with dextrin or starch, the effect on the blood sugar was much less marked than with equivalent quantities of glucose. Although with dextrin there was no increase in the sugar either of the blood or of the urine, this latter fluid contained an abundance of polysaccharide, as indicated by the very great increase in reducing power after hydrolysis. It is considered that it was not really dextrin that was absorbed by the blood, but some unusable carbohydrate, in other words, some impurity.

Experiments with *fructose* are of interest, since it is commonly believed that the assimilation limit (p. 217) for this sugar in normal individuals is substantially the same as for glucose. By feeding 200 gms., however, the remarkable result was obtained, that there was *no increase* in the sugar of the blood but a decided one in that of the urine, and this was not due to the presence of fructose, but, the authors believe, to the presence of reducing decomposition products of this sugar. In support of this view it is pointed out, first, that the increased excretion lasted for several hours following the ingestion of the sugar, and secondly, that fructose that had been partially decomposed by heating a solution of it until the sweet taste had almost disappeared caused a great increase in the sugar eliminated by the urine, along with pronounced symptoms of intestinal irritation. In terms of the commonly accepted doctrine of the glycogenic function of the liver, the failure of fructose to cause an increase in blood sugar would be attributed to its retention as glycogen by this viscus, but Folin and Berglund could readily detect fructose in the blood serum, although, as already remarked, none appeared in the urine. They interpret these differences by assuming that sugar, after its addition to the blood, is removed as glycogen only in part by the liver, the remainder being taken up by other tissues, such as the muscles. The glycogen-retaining power of the liver is probably the same for glucose and fructose, so that, after ingestion of equal quantities of these sugars, similar proportions will escape through the liver and become added to the systemic blood, in which, however, the concentration of fructose will not rise significantly because the tissues immediately absorb it. On the other hand, they will not absorb glucose so readily because they already contain a considerable percentage of this sugar. This interpretation harmonises well with the idea that a certain tension of glucose is present in the tissues (p. 303).

Since galactose and its disaccharide lactose form glycogen much less readily than glucose or fructose, it became of interest to investigate the influence of those sugars in the same manner. Ingestion of more than 20 to 40 gms. of galactose caused a decided increase in the urinary sugar without any significant change in blood sugar, indicating that it had been immediately absorbed by the tissues, which do not contain galactose. There is apparently no renal threshold for galactose, and in this regard it differs from fructose. It was also observed that the

retention and utilisation of galactose by the human organism depends very largely on the quantity of available glucose ; thus, when galactose was given, together with glucose, its excretion was less than one-tenth as great as the excretion following the ingestion of an equal quantity of galactose alone. This observation would seem to explain why milk sugar is a suitable food-stuff for the young animal, although of only a low assimilative value for adults. It may be that its value for the young is dependent on the fact that it is used in the building of nerve tissue.

It is considered possible that the results of Benedict, etc., may have been due to the escape into the urine of various unusable carbohydrate decomposition products, and it is of practical importance to remember that these foreign products may sometimes be excreted in sufficient quantities to give positive clinical tests for sugar in the urine. Such foods as confections, preserved fruits, canned vegetables, as well as grains and breadstuffs, must contain them. Whether their absorption has any deleterious effect on the organism is unknown.

To summarise Folin and Berglund's results, the ingestion of glucose causes decided hyperglycæmia, but no increase in urinary sugar, until a renal threshold for this substance is reached. Galactose, on the other hand, causes no increase in blood sugar, although some immediately escapes into the urine, in which fluid the concentration of sugar may rise so as to cause glycosuria. With fructose there is also no increase in blood sugar, although it does not, like galactose, immediately leak into the urine, since there is a renal threshold for fructose as for glucose. There seems little doubt, therefore, that the idea of the renal threshold for glucose, and probably also for fructose, originally propounded by Claude Bernard, is a correct one. Such a threshold, however, does not exist for other sugars, or for decomposition products of sugars, so that these readily appear in the urine, and are the cause for the slight, but varying, reducing power described by Benedict and Osterberg.

Folin and Benedict offer a chemical explanation for the renal threshold. They assume that the concentration of glucose in the tissues is the same as in the blood (this we would designate as the glucose tension), and that its level can be raised to a certain extent without the sugar being immediately converted into glycogen. This implies that this glucose tension in the tissues is submaximal, so that when more glucose is added to the blood

there is some room for it in the tissues into which it migrates, although not as quickly as would have been the case if no glucose were present in them. When the glucose-holding capacity of the tissues has reached its limit, glycogen formation begins, but since this is a slower process than that of the simple absorption of glucose, the latter begins, as it were, to back up in the blood, with the result that the kidneys are called upon to get rid of the excess. At this stage then glycosuria begins, and the sugar excretion, once begun, continues until the blood sugar has become reduced below the normal level. When galactose, or, to a less extent, fructose, is taken, the blood sugar does not become increased, because there is a complete vacuum in the tissues for these sugars. At the same time, they appear in the urine because there is no renal threshold for them. This interpretation of their results would harmonise with the conclusion we have drawn regarding the mechanism of the action of insulin, with the difference that we believe that the sugar which disappears into the tissues very quickly becomes formed into some unknown complex with which glycogen stands in a certain equilibrium, and from which it is impossible to recover sugar by hydrolysis. Further references to this hypothetical compound will be found on page 255.

Assimilation Limits.—Under this somewhat vague title are included a number of conditions. "*Carbohydrate tolerance*," a term originally used in connection with diabetes, means the amount of carbohydrate, from whatever source, which a patient can tolerate without any glucose appearing in the twenty-four hour urine as judged by the clinical tests. "*Limit of sugar assimilation*" means the amount of sugar which can be taken in one dose without causing glycosuria. As usually applied, 100 gms. of glucose is given either before or after breakfast, and the urine then tested at intervals. For obvious reasons, which have already been sufficiently discussed, this test can be of little diagnostic value. Closely linked with this idea is that of *alimentary glycosuria*, in which it is assumed that sugar taken in excess of the assimilation limit will reappear quantitatively in the urine. It has now been known for many years (Linossier and Rogue, 1895) that this is far from being the case since only a small fraction of the excess is actually excreted by normal individuals. Indeed, as has been pointed out, particularly by Allen (1913),

the more sugar that is given, the more is utilised, there being, indeed, no limit to the extent to which this can occur in the normal individual. To indicate this increasing disappearance, the expressions "*coefficient of utilisation*" and the "*paradoxical law of dextrose*" have been introduced.

It is to Woodyatt, Sansun, and Wilder (1915) that we owe the clearest statement of what is really meant by utilisation of glucose. These observers found, in both man and laboratory animals, that glucose can be injected continuously into the vein at the rate of about 0·85 gm. per kilo per hour without causing glycosuria. This represents, therefore, the tolerance limit, and between it and 2 gms. of glucose per kilo per hour a certain fraction of the ingested sugar reappears in the urine, varying with different individuals, and in the same one, with varying rates of injection. Usually, with the continuous ingestion of twice as much sugar as the utilisation figure, about 10 per cent. is lost in the urine, and with four times as much, the loss may rise to 35 or 40 per cent., beyond which, however, no further increase of sugar in the urine occurs. It is interesting, further, to note that, according to Woodyatt, the rate of absorption of glucose from the alimentary canal never exceeds 1·8 gms. per kilo per hour, so that an excretion of about 10 per cent. of absorbed glucose is the maximum in the normal individual that could be obtained on taking glucose by mouth. Since, however, this maximum could last for only a short period of time, a much lower percentage of sugar would appear in the urine, even when very large amounts of glucose were ingested. This corresponds to the maximum rate of intestinal absorption of glucose for rabbits found by E. L. Scott and Ford (p. 206).

These considerations will make it evident that the determination of the so-called assimilation limit can be of comparatively little value in the diagnosis of early cases of diabetes, unless when all the conditions referred to have been considered.

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CHAPTER XV.

ENDOGENOUS HYPERGLYCÆMIA (EXPERIMENTAL DIABETES).

Endogenous hyperglycæmia may result from: (1) Nervous conditions; (2) the effect of certain hormones, especially epinephrin; (3) asphyxial conditions; (4) the effect of drugs, especially narcotics and anæsthetics. Sometimes a given agency may belong to more than one of these groups, thus the anæsthetics may cause some degree of asphyxia, and at the same time have a hyperglycæmic influence of their own. We will consider each of these in the order given, and then see in how far they may be interdependent of one another.

Nervous Hyperglycæmia.—In 1857 Claude Bernard discovered that puncture of the fourth ventricle with a needle, in well-fed rabbits, was soon followed by marked glycosuria lasting for several hours. This led to a long series of experiments, by Bernard and others, from which it was concluded that the puncture acted by causing the stimulation of some nerve centre (glycogenic centre) concerned in the control of the breakdown of glycogen in the liver.

Eckhard (1869) showed that electrical stimulation of sensory nerves, particularly the vagus, could also cause glycosuria, and the view became developed that the blood sugar level is controlled through the nervous system. Pflüger (1903), for example, suggested that it is by reflex action that the glycogen in the muscles is replaced when it becomes used up as a result of contraction. According to this writer the contractions act on (compress) the muscle spindles, setting up afferent impulses which are transmitted to the glycogenic centre, with the result that increased hepatic glycogenolysis occurs. It is, of course, also possible that the receptors of this hypothetical reflex are excited by chemical substances produced as a result of contraction in the muscles, and it may be that these, instead of acting locally, really act as hormones by being conveyed with the blood to the liver. This general outline will serve to indicate the significance of a study of the nervous hyperglycæmias, of which we may distinguish those due to:

(1) irritation of the centre itself—piqûre; (2) irritation of afferent nerves; (3) irritation of the efferent pathway.

Piqûre.—This experiment is most conveniently performed on the rabbit.

Under local anæsthesia (ethyl chloride), the head is grasped firmly in the left hand and bent forward and a moderately sharp trochar is then pushed through the skull, just in front of the protuberance in the direction of the outer canthi, until it is stopped by the basilar process. This punctures the medulla somewhere between the roots of origin of the eighth and tenth nerves, and there is considerable latitude in the effective area. Because of wounding of the vermis of the cerebellum in this operation, forced movements are usually set up, and to avoid these, as well as make more certain of the position of the puncture, Eckhard, and more recently Stewart and Rogoff (1918), have recommended that the occipito-atlantoid membrane be laid bare, under local anæsthesia, and incised, after which, by bending the head forwards, the floor of the fourth ventricle can readily be seen. In our experience, the puncture through the skull is quite satisfactory, and is to be preferred, since some hæmorrhage is difficult to avoid in the Eckhard operation.

In well-fed rabbits the blood sugar rises very rapidly following the puncture, and it may reach to three or four times the normal level within an hour. It then either begins to descend slowly or remains elevated for some time, finally falling, so as to regain the normal level, usually in six or eight hours, although sometimes it may remain well above the normal, even after twenty-four hours. Representative curves are shown in Fig. 19, page 223. In the urine voided within an hour of the piqûre, considerable sugar is usually present, and in three hours this may reach a percentage of eight, or higher. A certain degree of diuresis usually accompanies the glycosuria, and if the urine be scanty, little sugar may be present in it, notwithstanding the blood sugar is high.

The question arises as to whether the lesion caused by the puncture acts by destroying some centre having a tonic inhibitory influence over the breakdown of glycogen, or by stimulating one which excites this process? Most of the evidence favours the latter possibility. Thus, piqûre is said to be relatively ineffective in completely anæsthetised animals, although this is denied by Neubauer (1912), who used urethane and by Borberg (cf. Bang), who used ether. It is also stated that the effects may pass off in a few hours, before all of the glycogen has been

used up, and that a second puncture may again cause hyperglycæmia and glycosuria in animals which have recovered from a preceding puncture, and in which the blood sugar has returned to normal. It might appear that some light could be thrown on this question by applying electrical stimulation to the medulla, but I am not aware that this has been done in such a way as to avoid the serious respiratory disturbances which may in themselves cause hyperglycæmia (p. 234). The available evidence favours the view that the puncture leads to the development of injury processes (e.g. the appearance of acid substances) which act as stimuli for neighbouring uninjured nerve centres.

The Relationship between the Effects of Piqûre and the Glycogen Content of the Liver.—Based on the researches of Claude Bernard, it is believed that the results of piqûre are dependent upon the presence of a certain percentage of glycogen in the liver. In more recent work, in which the behaviour of the sugar in the blood, rather than that of the urine, was observed, this relationship has in general been confirmed, although the blood sugar has been found to return to near the normal level while some glycogen still remained in the liver, which may have been due to cessation of irritation at the site of puncture.

Thus, Stewart and Rogoff (loc. cit.) report two rabbits in which piqûre failed to cause any decided degree of hyperglycæmia (Nos. 187 and 157), with percentages of glycogen in the livers of 0·34 and 0·057 respectively. In both of these animals asphyxia was also incapable of causing the blood sugar to increase. That the hyperglycæmia may begin to diminish before all the glycogen has disappeared from the liver is illustrated in one observation in which the blood sugar reached its maximum of 0·457 per cent. in about two hours following piqûre, then fell rapidly so as to reach 0·187 per cent. in about five hours. It remained about this level until next day when, twenty-five hours after the piqûre, it was still 0·170 per cent., and 2 per cent. of glycogen was found present in the liver. In another case, 0·6 per cent. of glycogen remained in the liver in seventeen hours following piqûre, the blood sugar, in a little over two hours being 0·400 per cent. and in nine hours 0·200 per cent.

The Effect of Insulin on Piqûre Hyperglycæmia.—This is very striking, as shown in the curves of Fig. 19, in which the effects of piqûre on five normal rabbits are contrasted with those on three that were previously injected with insulin. Insulin prevents the hyperglycæmia, either because it inhibits the ex-

citation of the glycogenolytic process, or because it causes the excess of sugar discharged into the blood to disappear as quickly as it is formed, or because both of these processes come into operation. We have, so far, no direct evidence as to which of them is primarily at work, but it is of some significance that more glycogen was found, after piqûre, in the livers of two insulin-treated

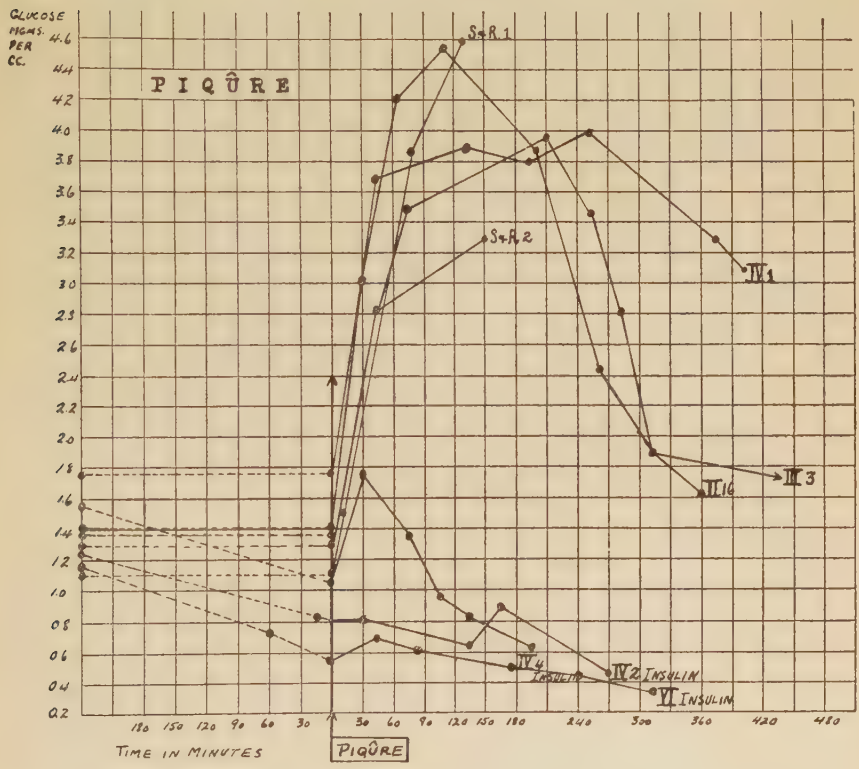

FIG. 19.—Curves showing effects of picûre on normal and on insulin-treated rabbits. Ordinates—per cent. blood sugar. Abscissæ—time in minutes. (From Banting, Best, Collip, Macleod, and Noble.)

animals than in those of two without insulin (viz., 4·4 and 2·64 per cent., as compared with 0·59 and 2·0 per cent., respectively). There can be little doubt, from its effect in exogenous hyperglycæmia, that insulin also causes the excess of sugar which appears in the blood after piqûre to migrate more rapidly into the tissues.

Efferent Nerves.—Disregarding for the present the possibility

that no glycogenic centre really exists, but that the hyperglycogenolysis following piqûre is merely the result of the respiratory and circulatory disturbances occasioned by stimulation, or destruction, of the medullary centres, we shall proceed to examine the evidence which is considered to prove that efferent glycogenolytic impulses are transmitted to the liver through certain nerves. The vagus (bulbo sacral autonomic) and the splanchnic nerves (thoracico lumbar autonomic) are the possible pathways.

Claude Bernard showed that after section of the vagi piqûre was effective in causing glycosuria, whereas Eckhard found that it was not so after section of the spinal cord above the first thoracic root. Both these workers also found piqûre to be ineffective after section of the great splanchnic nerves, and the conclusion was drawn that it must be by this pathway that the efferent impulses travel. The experiments do not, however, justify such a conclusion, since the fall of blood pressure resulting from the severance of the vaso constrictor pathway would, in itself, so depress the excretion of urine as to prevent sugar appearing in it, even although hyperglycæmia might be present. Some of the earlier experimenters also attempted to locate the efferent pathway in the spinal cord by electrical stimulation at various levels, and Schiff (1859), concluded that glycosuria can be produced by stimulation anywhere from the base of the brain (Hirnschenkel) to the place where the roots for the visceral nerves leave the cord. Macleod (1907) found that the blood sugar in etherised dogs rose above the anæsthetic level within thirty minutes after electrical stimulation of the lower cervical and the upper thoracic portions of the spinal cord, but that stimulation had no effect when applied below the ninth thoracic segment. At first sight this seemed to demonstrate the position of the efferent pathway, but further investigation showed that disturbance of respiration was mainly responsible for the hyperglycæmia, since no significant changes in blood sugar occurred when, by artificial respiration or the administration of oxygen, asphyxial disturbances were guarded against.

The many sources of confusion which are evidently incurred in experiments in which the spinal cord is exposed and stimulated in anæsthetised animals render it difficult to demonstrate any glycogenolytic nerve pathway here. On the other hand, such fibres can readily be demonstrated in the great splanchnic nerves. Thus, in 1894, the Cavazzani brothers found the sugar of the blood of the hepatic veins to become increased by stimulation of the cœliac plexus, and in 1906 Macleod found that of the arterial blood, in etherised dogs, to increase markedly when the splanchnic nerve on the left side was stimulated just after its entry to the abdomen. Glycosuria of marked degree soon

also became evident, accompanied by diuresis (Fig. 20). Much more striking results were obtained when the electrodes were applied to the uncut nerve than when applied to the peripheral end after cutting it, and it was found important to ensure an abundant supply of glycogen in the liver, by previously feeding the animals with bread and sugar. By stimulation of the uncut nerves, however, a certain degree of respiratory disturbance is not infrequent, probably because of stimulation of afferent fibres in the splanchnic nerves, so that here again the possibility of asphyxial hyperglycæmia must be considered. It was attempted to rule this out by the free administration of oxygen (by continuous perflation through a catheter passed down the

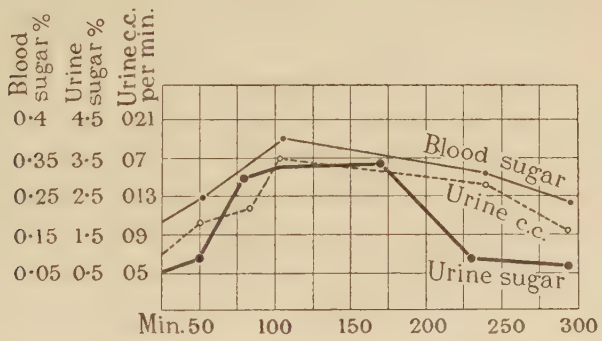

FIG. 20.—Curves showing relationships between blood sugar percentage and the amount of sugar excreted in the urine during splanchnic stimulation.

bronchi), and it is significant that, although hyperglycæmia still followed the stimulation, it was not so marked as without oxygen.

In the foregoing experiments the sugar was determined by the older methods of Waymouth Reid and of Rona and Michaelis, for which considerable quantities of arterial blood had to be used. More recently, since the introduction of micro methods, the blood has been taken directly from the vena cava opposite the openings of the hepatic veins, the cava being temporarily clamped above and below the liver during the moment of aspiration of the blood sample. The following table shows the average results of eight experiments using this method, and they leave no doubt that stimulation of the splanchnic nerve causes an increased discharge of sugar from the liver :—

PERCENTAGE OF SUGAR IN THE BLOOD FROM THE VENA CAVA

	Before Stimulation of the Splanchnic Nerve.	In from 5-10 Minutes after Stimulation.
Average . .	0·148 (eight observations)	0·190 (eight observations)
Minimum . .	0·111	0·110
Maximum .	0·199	0·280

(MACLEOD.)

By the use of similar methods it has been found that stimulation of the nerve fibres in the hepatic pedicle also cause an increased discharge of sugar. In one experiment the percentage of blood sugar rose from 0·150–0·294 after stimulation for six minutes, in others, from 0·165–0·204 after seven minutes and from 0·125–0·160 after eight minutes. Similar results following stimulation of the hepatic nerves have also been obtained by examining the arterial blood, although, as already remarked, the time intervals necessary to show the changes are much longer.

Although these observations yield results which are most simply interpreted by assuming that glycogenolytic nerve fibres are involved, there yet remains the possibility that they are secondary to changes in general blood pressure, or to reflex effects on respiration. To rule out these possibilities the nerve fibres in the hepatic pedicle were completely severed before stimulating the splanchnic nerves. In five experiments of this nature no increase in blood sugar was found to occur. Since in these experiments the usual degree of change in blood pressure would occur, as well as reflex respiratory disturbances, the results are strong evidence for the presence of glycogenolytic fibres in the splanchnic and hepatic nerves.

The effect of insulin has not been studied in this form of hyperglycæmia, because of the experimental difficulties. Anæsthetised animals must obviously be used, and this in itself makes the experiment one of doubtful value.

Epinephrin Hyperglycæmia.—Blum was the first to show that injection of epinephrin (adrenalin, adrenin) usually causes glycosuria. It soon became evident that a rich deposition of glycogen in the liver favoured this result, although it was found that epinephrin can usually cause some glycosuria, even when given to starved animals (Pollak, 1909; Ritzmann, 1909; Neubauer, 1912; and Bang, 1913).

Most authors are agreed that epinephrin causes diminution of glycogen of the liver. According to Bierry and Gatin-Gruzewska (1915), it is, indeed, a certain agency by which both the liver and muscles of the animal can be freed of glycogen, although other observers, for example Drummond and Noel Paton (1904), were able to show, in fed rabbits, that no decided diminution in glycogen content occurred when the epinephrin was injected in slowly increasing dosage from time to time. Rolly has confirmed this observation. Ritzmann (1909), after demonstrating that a relationship exists between the amount of epinephrin injected and the incidence of glycosuria in cats and rabbits, found this also to be the case between the amount of glycogen deposited in the tissues and the glycosuria. For example, in a glycogen-rich animal an adrenalin solution of 1-1,000,000 caused a considerable amount of sugar to appear, whereas in another animal, which was practically glycogen-free, this concentration had no glycosuric effect. Differences in this, dependent on the concentration of epinephrin in the blood, have also been demonstrated by Bang and Pollak. Given intravenously epinephrin seldom causes glycosuria (Pollak), partly because the hyperglycæmia is only transient and partly because there is interference with the excretion of urine by the kidney. When a diuretic is given along with the epinephrin, glycosuria becomes a prominent symptom and the hyperglycæmia much more lasting.

Using the subcutaneous pathway, injections of equal quantities of epinephrin into well-fed rabbits produce about the same degree of hyperglycæmia, and based on this fact, Eadie and Macleod (1923) attempted to develop a method for the assay of insulin, by ascertaining the extent to which varying quantities of this hormone would prevent the rise of blood sugar following injection of equal amounts of epinephrin (p. 230). A similar method had previously been employed by Zuelzer (1907) to measure the strength of the pancreatic extracts (see p. 57) prepared by him.

Although a general relationship between the glycogen content of the liver and the hyperglycæmic effects of epinephrin is supposed to exist, further examination has revealed some very curious and important facts. Thus, Pollak has found that when the glycogen has been deposited by feeding glucose to previously starved rabbits it disappears, following the injection of epinephrin, much more readily than when it has been deposited by feeding the animal with fructose. These differences become apparent only when small quantities of epinephrin are injected, and they indicate that the glycogen formed from fructose is more resistant than that formed from glucose. This fact has

an interesting bearing in connection with diabetes, in which it is known that fructose can be better tolerated than glucose (see p. 46). Neubauer has also observed that in phosphorus poisoning glycogen can still be formed in the liver when fructose or cane sugar are administered to the animal, although none is formed with glucose.

A most important fact, first demonstrated by Pollak and confirmed by Rolly, is that after all glycogen has disappeared from the liver, administration of epinephrin still causes an increase in blood sugar, which may result in glycosuria. Pollak states that as much glycogen may be formed in the liver of previously starved animals by injecting epinephrin as by feeding with carbohydrate-rich foods, indicating that a new formation of glycogen (gluconeogenesis) must be occurring. No data are given which would indicate the source of this new sugar, but Bang refers to researches in which examination of the nitrogen excretion showed that the source cannot be exclusively protein. This, therefore, suggests the possibility that the glycogen comes from fat or, as Bang supposes, from some intermediary substance of carbohydrate metabolism which has not as yet been identified. Further evidence that such an hypothetical intermediary substance may exist is afforded by experiments in which the tissues have been examined for glycogen and sugar after the intravenous injection of sugar (p. 315), and also by those bearing on the action of insulin (p. 178).

The following results by Markowitz illustrate the large amounts of sugar which may be added to the blood, and of glycogen which may be deposited in the liver in starved rabbits, following the repeated administration of epinephrin.

In thirteen rabbits all deprived of food for five days or longer, and subsequently repeatedly injected with epinephrin for varying periods up to five days, the following percentage amounts of glycogen were found in the livers: 0·33, 0·16, 0·98, 1·05, 0·34, 1·67, 0·152, 2·77, 1·45, 1·27, 1·09, 0·031, 1·12. The percentage of blood sugar, in another group of rabbits, rose on the last day of epinephrin injection to the following levels: 0·113, 0·194, 0·412, 0·407, 0·047, 0·446, 0·382, 0·305.

Similar production of glycogen may also occur, in animals previously deprived of this substance, by starvation (p. 140), or by strychnine, or as a result of the administration of narcotics, or

as a result of infection (Hirsch and Rolly). It is of significance that excessive protein breakdown also occurs after epinephrin and during infections, although it does not occur with narcotics.

With regard to the mechanism by which epinephrin acts on the glycogen of the liver, it may be stated that Snyder, Martin, and Levin (1922) have shown that its effects on the perfused turtle liver are related to the pH of the perfusion fluid, and they believe that the supposed direct influence of epinephrin on the glycogenolytic process is dependent on changes in volume flow, which vary with the pH of the perfusion fluid.

The Effect of Insulin on Epinephrin Hyperglycæmia.—When insulin and epinephrin are injected simultaneously, the latter hormone acts more quickly in producing hyperglycæmia than the former does in preventing it, but if the insulin is given about one hour (in rabbits) prior to the epinephrin, the blood sugar may remain practically unaffected. Although, in their effects on blood sugar, the two hormones may therefore be said to be antagonistic, it must be remembered that this may be of little physiological significance, and merely be due to the fact that the insulin leads to the disappearance from the blood of the excess of glucose which the epinephrin causes to be liberated from the liver, just as it does when the excess of glucose is absorbed from the intestine, or administered parenterally. As a matter of fact, we shall see that insulin and epinephrin both act similarly on the inorganic phosphates of the urine, and both cause, in dogs at least, an increase in the respiratory quotient and the calorie output. They are not antagonists in the sense that one prevents the other from acting.

Nevertheless there are several reasons why the apparent antagonism between insulin and epinephrin in connection with the blood sugar is of interest, and among these may be mentioned :—

(1) It may be possible to measure the strength of insulin by finding how much would be required to prevent the rise in blood sugar due to a definite amount of epinephrin, which in itself is readily standardised (Eadie and Macleod).

(2) The stimulus responsible for recovery of the blood sugar after it has fallen as a result of insulin, may be associated with an increased discharge of epinephrin from the adrenal glands (W. B. Cannon).

(3) Epinephrin may be useful as a clinical antidote in cases of excessive hypoglycæmia.

(4) The hyperglycæmia which follows removal of the pancreas may be due to uncontrolled action of epinephrin (Zuelzer, Hédon, Mayer, etc.).

The Assay of Insulin Based on its Effects on Epinephrin Hyperglycæmia.—This method of assay was originally suggested by Zuelzer (1907) for extracts of pancreas prepared by him. Eadie and I (1923) investigated its possible value by injecting into previously starved rabbits varying doses of insulin, and then, at an interval of one hour and a quarter, also injecting into each animal equal quantities of epinephrin. The blood sugar was determined in samples of blood taken just before the epinephrin, and again in one and a half, two, and three hours after it. The extent of the rise in blood sugar above the level existing immediately before injecting the epinephrin was then plotted against the amount of insulin injected, with the results shown by the crosses in Fig. 21.[1] It can be seen that no simple relation holds between the dose of insulin and its effect in preventing epinephrin hyperglycæmia, the small doses having relatively much greater influence than the large ones, which is also the case for the glucose equivalents of insulin as determined by Frank N. Allan (1924) (p. 96), and for the values of a in the assay equation, as determined by Macleod and Orr (p. 344).

When the logarithms of the doses of insulin are plotted against those of the rises in blood sugar the majority of them fall along a straight line. It was attempted to find an empirical formula for the curve. This was as follows :—

$$\frac{\text{Log } 20\,d}{2{\cdot}38} + \frac{\text{Log } r}{3{\cdot}84} = 1$$

and it will be seen that the actual observations fall tolerably close to the calculated curve, the greatest divergence being when small doses are used.

Since the increase in blood sugar following epinephrin will depend to a certain extent on the amount of glycogen deposited in the liver, and on other physiological factors, such as the amount of insulin already present in the body, it has not been

[1] The blood sugar in two hours after epinephrin are chosen for this curve since they were found to be the most constant.

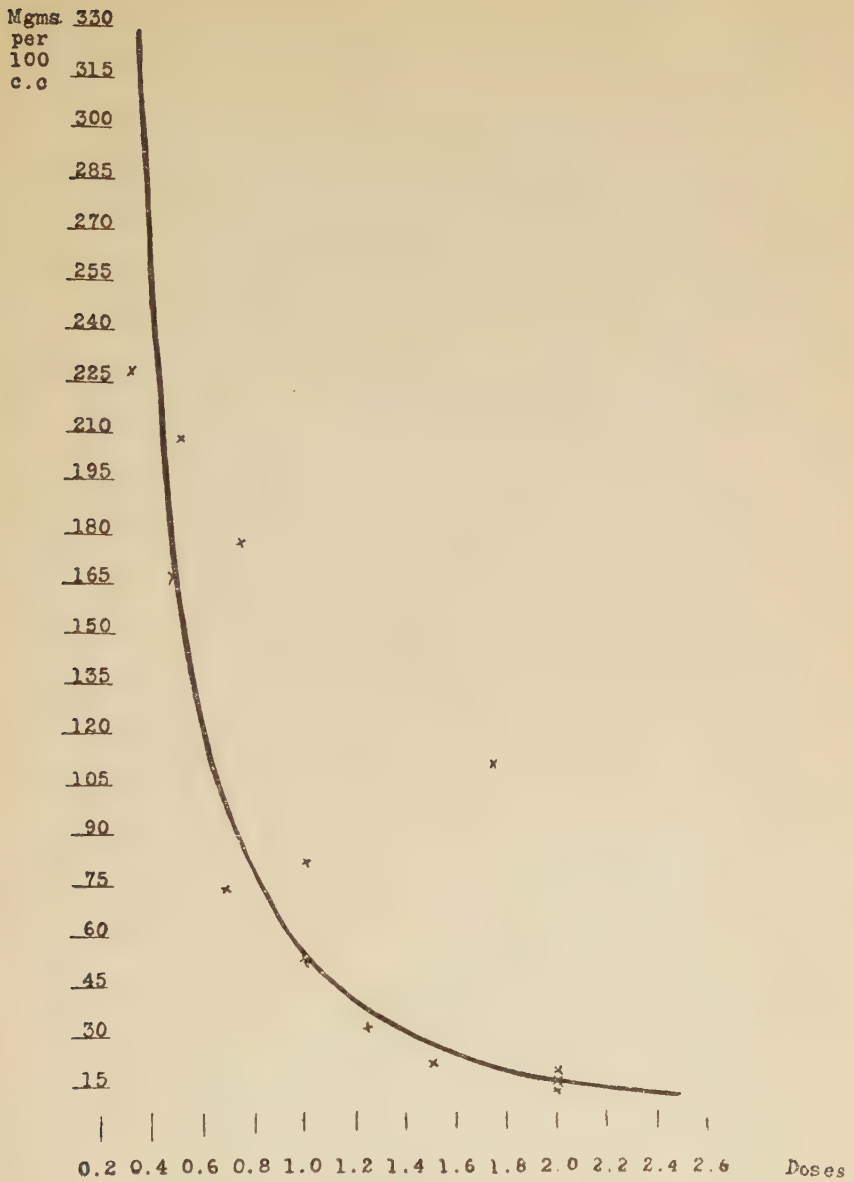

FIG. 21.—Curve showing relationships between units of insulin (abscissa), and the extent to which the hyperglycæmia caused by epinephrin was suppressed (ordinates). (Eadie and Macleod.)

found that the assay of insulin can be accurately carried out by this method.

Possible Relationship of Epinephrin to the Recovery from Insulin Hypoglycæmia.—The suggestion, that the beginning of the recovery in blood sugar following insulin is associated with a hypersecretion of epinephrin, harmonises with the fact that many of the symptoms occurring during the hypoglycæmia are not unlike those which follow injections of this hormone (the dilatation of the pupils, the sweating, the acceleration of the heart, etc.). Such a hypersecretion would also be favourable to the rise in blood sugar which usually occurs in glycogen-rich animals. Cannon, MacIver, and Bliss find support for this view in the fact that they have observed, in cats from which the adrenal glands have been removed, that after the lowering of the blood sugar due to insulin, there was no temporary wave of recovery, such as often occurs in normal animals (p. 272). They consider that this transient wave of recovery is associated with an increased discharge of epinephrin. On the other hand, they found that insulin caused convulsions with much greater frequency in cats with one adrenal gland excised and the other denervated, than in normal animals. Their evidence that epinephrin is secreted at a certain critical low level of blood sugar was obtained by observing the pulse rate after denervating the heart. With the adrenals intact acceleration occurs when the blood sugar is lowered to anywhere between 0·110 and 0·070 per cent., but no acceleration occurred in animals in which one adrenal was excised and the other one denervated.

It is also stated by Lewis (1923) that insulin is much more fatal in the case of adrenalectomised rats than in normal ones, and by Sundberg (1923), that rabbits with the adrenal medulla destroyed are more susceptible. These results may, however, depend on the general depression of the operation itself for, as Stewart and Rogoff have shown, insulin has exactly the same influence on rabbits adrenalectomised by their methods (p. 233), as on normal animals, and they could obtain no evidence that the amount of epinephrin in the blood of the adrenal veins becomes increased as a result of insulin.

Boothby and Rowntree have also concluded that a hypersecretion of epinephrin occurs during insulin hypoglycæmia, their evidence being based on the increase in oxygen consumption which is commonly observed (p. 257).

Epinephrin as an Antidote for Insulin.—As a clinical antidote for hypoglycæmic symptoms epinephrin has its place, particularly in cases where it has become impossible, through loss of consciousness, to give sugar by mouth. It is useless as a restorative of blood sugar, unless the liver contains glycogen, which in untreated cases of severe diabetes is unlikely to be the case. Whenever consciousness is regained, sugar should be given by mouth, since the effect of epinephrin soon passes off. Care must also be taken not to give enough to cause shock.

The Suprarenal Hypothesis of Pancreatic Diabetes.—Attractive in many ways though this hypothesis may be, it cannot be said to be supported by convincing evidence. Stewart and Rogoff (1918, 1920, 1922, 1923) have shown that the earlier researches of Zuelzer, Mayer, Frouin, and Hédon and Giraud, in which it was claimed that hyperglycæmia and glycosuria did not occur in animals from which both the pancreas and the adrenals were removed, are inconclusive, because of the condition of shock into which the animals are brought by the double operation.

To test the hypothesis properly, as they point out, it is necessary to devise means by which the animals can be kept alive in a tolerably normal state for a few days, following the removal of both glands. Even in the very carefully conducted experiments of Hédon and Giraud, this was not achieved. These workers extirpated the adrenals in dogs from which all of the pancreas had previously been removed, except for a graft which had been transplanted under the skin of the abdomen. On removal of the graft, immediately following the adrenalectomy, it was found that the blood sugar only rose slightly, and that it fell below the normal as the animal gradually became moribund, death occurring after a period of some seven hours.

It is now recognised that the quickly fatal issue of double adrenalectomy depends mainly on the removal of the cortex rather than the medulla of the gland, which latter is, however, the source of epinephrin. Taking advantage of this fact, Stewart and Rogoff have repeated these experiments by excising one adrenal and removing the other from the possibility of producing epinephrin, by completely cutting all its nerves and also destroying the medulla by means of a curette. After this operation, the animals live in a tolerably normal condition, although they lose in weight. In one of them, fifty-one days after the operation, the blood sugar being 0·096 per cent., the pancreas was removed, with the result that marked hyperglycæmia developed (0·198 per cent. in five-and-a-half hours, 0·286 per cent. in two days, and 0·278 per cent. in six days following the pancreatectomy), accompanied by glycosuria.

The animal lived for a week after the pancreatectomy, consuming daily large quantities of meat. In another experiment yielding similar results, the remaining (curetted) adrenal was excised five days after the pancreatectomy, but even in eighteen-and-a-half hours after the operation, the blood sugar was still 0·288 per cent. After this it began to fall, reaching 0·08 per cent. in forty-eight hours after the adrenalectomy. The animal died of collapse in sixty-seven hours when the blood sugar was 0·054. As would be expected, the excretion of urine became greatly curtailed as collapse developed, and the percentage of sugar in it much reduced.

In considering the mechanism of the antagonistic effect of insulin on epinephrin hyperglycæmia, the same possibilities are to be considered as in the case of piqûre, namely, a diminution of the glycogenolytic effect, and a more rapid disappearance of sugar from the blood. Evidence that the former is, at least partly, responsible has been obtained by Noble and O'Brien by finding that there is more glycogen in the liver of animals given insulin and adrenalin than in those given adrenalin alone (p. 176), both groups of animals being similarly fed.

Asphyxial Hyperglycæmia.—That glycosuria may become established after asphyxia has been known since the times of Claude Bernard and Eckhard.

Between 1891 and 1894, Araki found that respiration in atmospheres deficient in oxygen caused glycosuria, excretion of lactic acid and increase in the sugar of the blood. It gradually came to be considered possible that glycosuria, caused by various other agencies, might also be due to asphyxia. Among these may be especially mentioned drugs affecting the oxygen-carrying power of the blood, such as carbon monoxide and those causing paralysis of the voluntary muscles, such as curare. Although Claude Bernard maintained that this poison owed its diabetic influence partly to the direct action on the liver cells, Schiff (1858), found that it did not cause glycosuria when artificial respiration with air or oxygen was applied. Seelig (1905) stated that the glycosuria caused by ether was also due to asphyxia, and could be removed by the administration of oxygen, and Underhill (1905) found this also to be the case for the hyperglycæmia caused by piperidin.

These researches demonstrate sufficiently that hyperglycæmia and glycosuria may be dependent on asphyxia, and in considering the subject further the following questions present themselves :—

(1) Is the glycosuria always dependent on hyperglycæmia? Since modern methods for blood sugar estimation have

come into use, it has been an easy matter to demonstrate that asphyxia, however produced, causes the blood sugar very quickly to increase, provided the glycogen stores are sufficient. This can be shown on anæsthetised animals. Thus, in a dog anæsthetised with ether, hyperglycæmia became evident after thirty minutes of intermittent asphyxia, and after two and a half hours the blood sugar rose to 0·42 per cent. After discontinuing the asphyxia the blood sugar usually continues to rise for about half an hour (anæsthetised dogs) and then gradually falls. In recent experiments in unanæsthetised rabbits, done in collaboration with McCormick, the blood sugar was found to rise even although the liver did not contain more than a small percentage of glycogen. This fact indicates that asphyxia is a particularly potent stimulus of hyperglycæmia, and on this account Stewart and Rogoff (1917) use it as a test to determine whether an animal is suitable for the demonstration of hyperglycæmia by other experimental means.

(2) Is the hyperglycæmia due to a deficiency of oxygen or to excess of CO_2? Araki considered oxygen deficiency as the essential cause, while Schiff, Tiffenbach, and Edie (1902) asserted that an excess of CO_2 is responsible. The most searching inquiry with regard to this question is that of Kellaway (1920), who observed, in unanæsthetised animals, that respiration, through a mask, of atmospheres deficient in oxygen caused increase in blood sugar much more readily than did those containing an excess of CO_2 without deficiency of oxygen. After allowing the animal to breathe 5 per cent. oxygen for eight minutes, he found the blood sugar to rise from 0·126 per cent. to 0·421 per cent. In another similar experiment 7·3 per cent. of oxygen raised the blood sugar only to 0·156 per cent. after two and a quarter hours. On the other hand, percentages of CO_2 below 5 could be tolerated, provided oxygen was maintained at about the normal level. One may draw the conclusion that in ordinary asphyxia, anoxemia is the most important factor in causing hyperglycæmia, but that excess of CO_2 also plays a rôle.

(3) Is the hyperglycæmia entirely dependent on an accelerated breakdown of glycogen in the liver? Stewart and Rogoff failed to observe any hyperglycæmia in two rabbits in which no glycogen was found in the liver after death. It is difficult, however, to arrive at an answer to this question by comparing the effects

of asphyxia on starved and well-fed animals, since, although no glycogen may be detected after death, it may have been present before the asphyxia was started. The problem has, therefore, been investigated by seeing whether asphyxia would cause hyperglycæmia after removal of the liver from the circulation, by establishing an Eck fistula and then ligating the hepatic arteries (Macleod). In six out of a total of seven experiments of this nature, asphyxia, induced by clamping the respiration tube, only caused very slight increase in the sugar of the arterial blood. In three other similarly operated dogs, curare administered until complete muscular paralysis occurred did not affect the blood sugar level. From these experiments two important conclusions may be drawn: (1) That the hyperglycæmia of asphyxia cannot be dependent upon a depression in oxidative processes in the tissues; and (2) that the glycogen of the muscles cannot serve as the source of the increased sugar production.

(4) Is the increased sugar production in the liver, following asphyxia, due to a direct effect through the blood, or to asphyxial stimulation of the nerve control, or to both these processes? Since it is well known that all of the cardinal nerve centres become stimulated in asphyxia, it is to be expected that the centre controlling hepatic glycogenolysis will also be involved. To demonstrate this, the method has been to remove the liver from nervous control, by cutting the fibres of the hepatic plexus. Out of eight experiments on anæsthetised dogs in which this was done, mechanical asphyxia caused hyperglycæmia in six, in two of which it was marked, and in the others was only of slight degree. In four experiments in which curare was used undoubted hyperglycæmia became established in all. In so far as it is allowable to draw conclusions from these results, it would appear that asphyxia is more potent to cause hyperglycæmia when the nervous control of the liver is intact. It is apparently only when the asphyxia is intensive, as after curare, that it can directly affect the liver cells, and there is evidence that the effect is due to a change in the pH concentration of the blood. As has been explained elsewhere, it has been found impossible to demonstrate, in extracts of liver, any difference in the action of glycogenase in normal as compared with asphyxiated animals. On the other hand, it is well known that the action of diastase is greatly accelerated by a slight increase in acidity, provided the

original reaction of the enzyme mixture was not already on the acid side of neutrality (p. 149). The accumulation of CO_2 in asphyxial blood, along with other acids due to the anoxemia, may therefore be responsible for the hyperglycæmic effect, by accelerating the breakdown of glycogen in the liver.

An attempt was made to demonstrate such an action of asphyxial blood in the following manner. Blood was removed from an asphyxiated animal into a flask filled with hydrogen, in which it was defibrinated and then transferred through glass tubing filled with hydrogen into other flasks containing liver pulp and defibrinated blood. The mixture was then incubated in the presence of hydrogen, and the rate at which glycogen disappeared from the mixture compared with that in other flasks containing similar quantities of liver and arterial blood in the presence of oxygen. After three hours incubation, glycogenolysis in the arterial blood was found to be 45 per cent. in one experiment and 48 per cent. in another; that in asphyxial blood being 42·7 per cent. and 48 per cent. respectively. In contrast with these results it was easy to show that the rate of glycogenolysis was much accelerated when the atmosphere in the incubated mixtures contained CO_2. The results did not, therefore, demonstrate that the asphyxial blood had any accelerating influence. It must be remembered, however, that the conditions in these experiments are fundamentally different from those maintained in the intact animal. In the experiments *in vitro* only a small unchanging amount of blood can come in contact with the liver, whereas in the body large quantities are continually circulating through this organ. Changes in hydrogen-ion concentration may also occur within the hepatic cell, without there being demonstrable changes in the blood itself.

In asphyxia, therefore, we may conclude that the glycogenic centre first of all becomes excited by the slight changes in hydrogen-ion concentration due to the increase in the tension of CO_2, but that, when the asphyxia becomes more intense and the hydrogen-ion concentration, therefore, still further increased, the glycogenic function of the liver also becomes directly stimulated. The muscular convulsions due to the asphyxia contribute to this effect, and it may be partly on this account that exhausting muscular exercise causes the glycosuria in diabetic patients to be aggravated. To what extent a hypersecretion of epinephrin may be a contributory factor will be discussed later (p. 245).

In how far the hyperglycæmia following stimulation of afferent nerves, administration of anæsthetics and narcotics, hæmorrhage, etc., may be due to asphyxia cannot definitely be stated, but it is important that this factor should be constantly

held in mind. I found, when anoxæmia was entirely prevented by intratracheal administration of oxygen, that stimulation of the central end of the vagus nerve, or of the spinal cord, did not cause any significant increase in blood sugar. Thus, in an average time of ninety minutes after the first application of electrical stimulation to the sciatic nerve, the blood sugar in eight dogs only rose to an average of 0·170 when oxygen was freely administered, whereas when this was not the case, as in seven other dogs, it rose in about an hour to an average of about 0·222. Similarly, with such drugs as coniin, nicotine, piperidine, and pyridine the hyperglycæmic effects are probably mainly dependent on interference with the respiratory mechanism (Underhill).

Anæsthetics and Narcotics.—Asphyxia is usually considered to be responsible for the hyperglycæmia associated with general anæsthesia, but some evidence has been presented by Tatum and Atkinson (1922) which shows that other factors are also concerned, and that the action is partly peripheral. Some investigators also believe that hypersecretion of epinephrin is responsible for the increase in blood sugar (Keeton and Ross, 1919), but this is denied by Stewart and Rogoff (1917, 1918). Fujii (1921) found that ether caused less hyperglycæmia after double splanchnotomy, but this result may depend on several factors. The degree of hyperglycæmia is approximately proportional to the intensity of the anæsthesia, at least with ether, and there is no doubt that asphyxia plays a role, since intratracheal administration of oxygen along with light ether anæsthesia only causes low degrees of hyperglycæmia. Morphine and urethane also cause pronounced increase in blood sugar, but a potent narcotic which does not have this effect, as shown by Page (1923) and Edwards and Page (1924), is isoamylethylbarbituric acid (Amytal), a fact which we have repeatedly confirmed.

The Effect of Insulin on Asphyxial Hyperglycæmia.—In animals (rabbits) under the influence of insulin the blood sugar scarcely rises as a result of asphyxia, or if it does so, soon returns to the low level at which it stood prior to the asphyxia. Curves illustrating these results are reproduced in Fig. 22, which are self-explanatory. Insulin also prevents the development of hyperglycæmia in poisoning by carbon monoxide.

The Effect of Insulin on the Hyperglycæmia due to Anæs-

thetics and Narcotics.—When ether is given to rabbits during the hypoglycæmia due to insulin the blood sugar only rises to a slight extent, if at all, and when it is given to rabbits in which ether hyperglycæmia is already present, it rapidly causes the blood sugar to fall (Fig. 23). Given to dogs in which the hyperglycæmia due to ether is already established, more insulin is

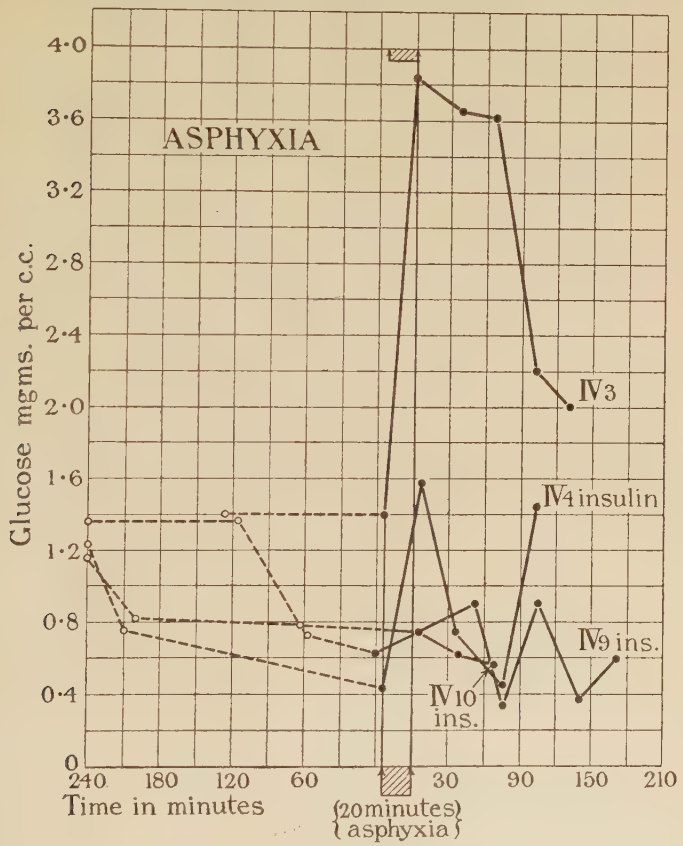

FIG. 22.—Curves showing the effects of asphyxia on the blood sugar of normal and insulin-injected rabbits. (Banting, Noble, etc.)

required to bring the blood sugar to within the normal limits than when the animals are given insulin prior to the administration of ether. This observation corresponds to the clinical experience that insulin is of much greater value, in diminishing the risks of surgical operations on diabetic patients, when it is given for some time prior to administration of anæsthetics, than when given only at the time of operation. This may partly be

because any, even a transient, degree of hyperglycæmia increases the surgical risks, but another and probably a more important reason is that the preliminary treatment with insulin causes glycogen to be deposited in the liver.

It is highly significant that insulin can scarcely cause any

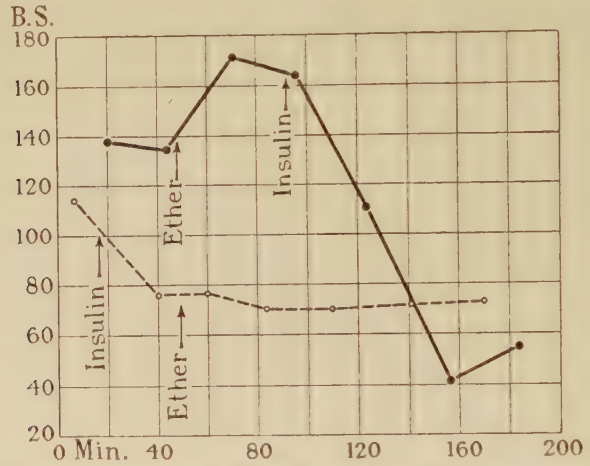

FIG. 23.—Curves showing effects of insulin when given before administering ether, and during ether narcosis. (Markowitz.)

lowering of the blood sugar in diabetic (depancreatised) dogs while under ether. This is illustrated in the following results, obtained by Hepburn, Latchford, McCormick, and Macleod (1924), on a dog five days after removal of the pancreas, and etherised about 10.30 A.M., blood for analysis being then removed at frequent intervals from the femoral artery.

Time.	Sugar per 100 c.c. Blood.	
	Femoral Artery.	Femoral Vein.
	mgm.	mgm.
11.12 a.m.	373	384
11.40 ,,	368	365
12.05 p.m.	383	Clot.
12.06 ,,	40 units insulin.	—
12.44 ,,	414	409
1.40 ,,	430	418
	60 units insulin.	—
2.40 ,,	460	442
2.47 ,,	60 units insulin.	—
3.35 ,,	472	443
3.40 ,,	60 units insulin.	—
4.55 ,,	450	430

(HEPBURN ET AL.)

In another similar experiment, in which 190 units of insulin were given in one dose, the blood sugar only fell in two hours from 0·329 per cent. to 0·302 per cent. There can be no doubt that insulin is practically without effect when given to depancreatised dogs after putting them under ether. Whether hyperglycæmia would fail to occur when ether is given to depancreatised animals injected some time previously with excess of insulin, remains to be investigated. It would appear that ether neutralises the action of insulin when there is no glycogen in the liver. The application of these observations in clinical practice is evident.

Stewart and Rogoff (1923) have shown that insulin can also prevent the hyperglycæmia due to morphine. It does not influence the general symptoms caused by this alkaloid, although in cats it prevents the rise in temperature which usually occurs.

The Supposed Relationship of the Adrenal Glands to Nervous Hyperglycæmia.—Blum, as a result of his discovery that epinephrin causes glycosuria, suggested that the glycosuria in *piqûre* might be due to stimulation of the adrenal gland. The close relationship of this gland to the sympathetic nervous system added support to this suggestion, and in 1906 Andre Mayer stated that piqûre failed to produce glycosuria after double adrenalectomy in rabbits.

Five years later, Kahn (1911), as the result of numerous experiments, concluded that the effects of piqûre and of splanchnic stimulation depend upon excitation of the adrenal gland, causing an abnormal secretion of epinephrin from the chromaffin substance. These researches do not inspire confidence, since no regard was taken of the many complicating factors which might explain the results, and which will be referred to immediately. Many of Kahn's proofs, moreover, are based on his finding that marked changes can be demonstrated in the chromaffin substance after piqûre and these, he assumes, must indicate an increased secretion of epinephrin, paying no regard to the possibility that such disappearance might depend, not upon an increased discharge of epinephrin, but upon its diminished production. He offered further evidence for his views in the fact that he could detect a higher concentration of epinephrin in the blood of the adrenal vein after piqûre than before it. The blood for this purpose, however, was not taken from the adrenal vein directly, but from the vena cava, and the presence of epinephrin was gauged by studying its vaso-constricting properties, without taking into account that, under the conditions of his experiment, pressor substances, other than epinephrin, might become

developed in it. As Stewart and Rogoff have pointed out, even if he had succeeded by this experiment in showing that epinephrin is increased in the blood, he did not show that this increase was sufficient to account for the marked hyperglycæmia which piqûre can so promptly bring about. The same criticism applies also to the work of Wakemann and Smit (1908), and to that of Borberg (cf. Bang), who, using Ehrmann's method, states that the amount of epinephrin in the blood of the adrenal vein becomes increased after piqûre. The support for the adrenalæmia hypothesis of piqûre offered by Jarisch (1914) cannot be accepted because of the unsatisfactory nature of the published results of his experiments. In view of the fact that the degree of hyperglycæmia following piqûre can vary considerably in different animals, notwithstanding the puncture is apparently in the proper position in the medulla, great care must be taken, not only to see that the liver in all the experimental animals contains an abundant supply of glycogen, but also that a sufficient number of experiments are performed to offset the variability in the results which are obtained, even in cases where there is sufficient glycogen.

In work of this type positive results are of very much greater value than negative ones; thus, if decided hyperglycæmia can be induced by piqûre in an adrenalectomised animal in which the liver contains large quantities of glycogen, the conclusion that the adrenal glands are not essential would seem to be established. Evidence of such a nature has been supplied by Stewart and Rogoff (1917).

One adrenal gland was first of all removed and then, sometime after recovery from the operation, the remaining gland completely denervated. Sometime after the second operation, and following upon a period during which large quantities of carbohydrate-rich foods were fed, piqûre was performed, under local anæsthesia by Eckhard's method. Blood sugars were taken before the piqûre and in from one to one-and-a-half, and two to two-and-a-half hours after it. Finally, the animal was asphyxiated until the heart became slowed, when a fourth sample of blood was removed, this being done in order to make certain that the animal was in a suitable condition to become hyperglycæmic. To illustrate the results, the following experiments may be cited. The right adrenal was removed on 19th November and the left one on 30th November. On 19th February, the rabbit being in excellent condition, normal blood sugar was found to be 0·12 per cent., and in two hours after piqûre 0·35 per cent., and in three-and-a-half hours after, 0·45 per cent. The animal was then asphyxiated for a period of about twenty minutes, with the result that the blood sugar rose to about 0·52 per cent. The liver contained 2·4 per cent. of glycogen and no accessory chromaffin tissue could be found on post-mortem examination. This is the most striking experiment, results of a similar nature being, however, ob-

tained in two other animals. It is true that in some of the adrenalectomised animals with fair quantities of glycogen no hyperglycæmia developed, either after piqûre or asphyxia, but Stewart and Rogoff consider these negative results as of no significance, since hyperglycæmia may also fail to occur, following piqûre or asphyxia in normal animals. Wertheimer and Battez (1910), Freund and Marchand (1914), Trendelenburg and Fleischhauer (1913), as the result of experiments that are apparently faultless, concur with Stewart and Rogoff in their conclusion that hypersecretion of epinephrin is not the cause of hyperglycæmia in piqûre. Incidentally it is to be noted that Stewart and Rogoff's results demonstrate beyond doubt that glycogen formation is little, if at all, affected by adrenalectomy.

It is evident that if hypersecretion of epinephrin were the immediate cause of increased glycogenolysis in the various forms of nervous hyperglycæmia, it should be possible to demonstrate that it is responsible for that occurring as a result of *stimulation of the great splanchnic nerve*. In this experiment, also, disturbances such as asphyxia and vascular changes in the general circulation are not incurred to the same extent as in piqûre. As a matter of fact, after excision of the adrenal gland stimulation of the splanchnic on the corresponding side is without effect on the blood sugar (Christie, Pearce, and Macleod, 1911), a result which, at first sight, would support the view that hypersecretion of epinephrin must be responsible for the hyperglycæmia obtained in animals with the adrenals intact. Further investigations by Pearce and Macleod showed, however, that stimulation of the splanchnic also failed to produce its usual effect after destruction of the nerve path between the adrenal glands and the liver, without injury to the adrenals or their nerve connections. This result would seem to warrant the conclusion that an increased secretion of epinephrin from the adrenal glands into the blood cannot *in itself* cause hyperglycæmia, Stewart and Rogoff having conclusively shown that such stimulation causes a distinct increase of epinephrin in the adrenal blood.

We are, therefore, face to face with two apparently contradictory results: (1) That stimulation of the splanchnic after adrenalectomy causes no hyperglycæmia; and (2) that stimulation of the splanchnic with the adrenal intact, but the hepatic nerves severed, is also without effect on the blood sugar. The presence of the adrenals, as well as the integrity of the nerve pathway, would both appear to be essential, and to explain such

a condition we must assume that the nervous control of the glycogenolytic mechanism is dependent upon the presence of a certain concentration of epinephrin in the blood. In order to test this possibility, the effects on the blood sugar of stimulation of the peripheral end of the hepatic plexus were compared before and after removal of the adrenal glands, and the results of typical experiments are shown in the accompanying table :—

INFLUENCE OF STIMULATION OF HEPATIC NERVES ON THE PERCENTAGE OF SUGAR IN PLASMA OF CAVA BLOOD.

Before Stimulation.	After Stimulation.	Time Interval between I. and II. (min.).
A. With adrenal glands intact—		
I.	II.	
0·153	0·179	6
* 0·165(17)	0·204	7
0·150(16)	0·294	6
0·125	0·160	8
0·233(15)	0·243	3
0·292(20)	0·372	8
0·425(14)	0·472	6
† 0·124	0·140	8
‡ 0·156	0·194	10
B. With adrenal glands removed—		
I.	II.	
0·155	0·149	8
0·133(20)	0·120	8
0·323	0·274	8
0·239(14)	0·225	8
0·141	0·145	9
0·137(16)	0·126	10
† 0·144	0·133	8
—	0·110	25
‡ 0·273	0·400	10
0·179	0·157	(10)

* The figures in brackets show the time (in minutes) elapsing since the nerve was stimulated, the observations on each animal being grouped together.

† and ‡. Each of these indicates that the same animals were observed before and after adrenalectomy.

With the adrenals intact, the blood sugar very quickly increased when the plexus was stimulated, but after their removal there was, with one exception, a slight decrease.

There are two objections to these experiments, the one, that the animals were anæsthetised; and the other, that the absence of effect after adrenalectomy may have been dependent, not upon the actual withdrawal of epinephrin from the blood, but upon the shocked condition into which the animal was brought by the operation. Stewart and Rogoff have rightly criticised all work in which conclusions are drawn from results obtained under these conditions, and we are well aware that their criticism is applicable in the foregoing experiment. On the other hand, we would point out that in our experiments the interval, intervening between adrenalectomy and stimulation of the plexus (thirty minutes), was probably not long enough to cause any profound degree of shock, although apparently it was so to bring about a decrease in adrenal concentration sufficient to diminish the conductivity in the nervous pathway.

The Relationship of Asphyxial and Anæsthetic Hyperglycæmia to Hypersecretion of Epinephrin.—It is stated that after adrenalectomy the blood sugar is not affected by either of these conditions, to anything like the same extent as in intact animals. Cannon and Hoskins (1911), Elliott (1912), and van Anrep and Kellaway (1912) have also presented evidence of an increased output of epinephrin from the gland as the result of asphyxia and anæsthetics, but Gley and Quinquad (1918) and Stewart and Rogoff do not consider the evidence as satisfactory. In support of their position, Stewart and Rogoff have published numerous experiments in which asphyxia and ether were found to cause distinct hyperglycæmia in rabbits in which the adrenal gland on one side was removed, and that on the other side denervated. Several observations have also been made on rabbits which had survived the removal of both adrenals, and in which, therefore, no questions can arise as to the entire removal of the source of epinephrin in the body. It is true that some accessory chromaffin tissue might still remain after such a double operation, but in face of the striking results obtained, it seems incredible that such small traces of tissue could yield a sufficient amount of epinephrin to account for the results which the following experiment will illustrate (Stewart and Rogoff).

The right adrenal was removed on 19th September, and the left on 21st October. On 25th November, when the animal was in excellent condition, the blood contained 0·13 per cent. of glucose. In twenty-five

minutes after starting light etherisation the percentage had risen to 0·16 per cent., and an hour later, etherisation being light in the interval, to 0·27 per cent. The animal was then asphyxiated to the extent that the heart became slowed, and in twenty minutes the blood sugar had risen to 0·37 per cent. The liver was found to contain 3·13 per cent. of glycogen.

Space will not permit of an analysis of the evidence furnished by the two sides to the controversy, as to whether or not excess of epinephrin is actually secreted into the blood in asphyxia and anæsthesia, but it seems clear that the adrenal gland is in some way associated. There is also no doubt that severe asphyxia, by raising the $H^{\cdot}$ ion concentration of the blood, can cause hyperglycæmia because of increased glycogenolysis (see p. 234).

Olmsted has recently contributed very clear-cut results by experiments on decapitate cats kept alive by artificial respiration. In such preparations the blood sugar is high immediately following the decapitation, no doubt because of the preceding deep anæsthesia, but it falls in the course of a few hours to about the normal level. If asphyxia be induced by interrupting the artificial respiration, marked hyperglycæmia quickly results. If, however, the adrenal glands be isolated, the blood sugar remains practically unchanged by the asphyxia.

The general conclusion which we would draw is that the adrenal glands in some way maintain the excitability of the glycogenolytic mechanism of the liver. How this acts we are not prepared to state.

Phlorhizin Diabetes.—In this form of experimental diabetes the blood sugar is little affected, although large quantities of sugar appear in the urine, and the respiratory quotient fails to rise in response to the ingestion of carbohydrate. The effect of insulin on these two symptoms in dogs kept fully under the drug was investigated by S. U. Page, but it was found by no means easy to obtain satisfactory data, especially in connection with the glycosuria, the reason being that the dogs were peculiarly liable to develop hypoglycæmic symptoms, because phlorhizin by itself tends also to cause a lowering of blood sugar in this animal. The respired air was collected in a counterpoised spirometer by using a mask and valves, and Table XX. shows the results of one of the experiments :—

TABLE XX.

No.	Date.	Time.	O_2 Inspired per hr.	CO_2 Expired per hr.	R.Q.	Remarks.
	1922					
Dog. 2	July 5	—	—	—	—	Phlorhizin started daily.
,,	,, 10	10.30 A.M.	3·40	2·40	0·70	——
,,	,, ,,	1.15 P.M.	3·31	2·35	0·71	40 gms. sucrose at 12 noon.
,,	,, 11	—	—	—	—	Insulin at 11·50 a.m.
,,	,, ,,	2.15 P.M.	3·52	3·20	0·91	40 gms. sucrose at 12·5 p.m.
,,	,, 12	10.30 A.M.	3·60	2·58	0·72	——
,,	,, ,,	2.15 P.M.	4·48	3·76	0·83	40 gms. sucrose at 1 p.m.
,,	,, 13	10.30 A.M.	3·72	2·70	0·73	——
,,	,, ,,	P.M.	2·90	2·45	0·84	40 gms. sucrose at 11·50 a.m.

Whereas within two hours after the ingestion of 40 gms. sucrose, no change occurred in R.Q. on 10th July, it increased to 0·91 on 11th July following the injection of insulin, and to 0·83 and 0·84 on 12th and 13th July respectively, indicating that its effect had lasted for this time. A similar prolonged effect of insulin was observed in another similar experiment on a different dog. In one animal, fed daily with equal quantities of lean meat and given phlorhizin so as to cause a moderate degree of diabetes (D : N ratio 1·8-1·9), the daily excretion of sugar for a period of four days was 21·24 grs., 21·96 grs., 20·84 grs., 22·14 grs., and on the next day it fell to 16·26 grs. as the result of the injection of sufficient insulin (18u) to bring the blood sugar just to the convulsive level.

More complete observations have been made by M. Ringer, by using the respiratory chamber of Graham Lusk. No food was given the dogs during the period of the observations, and the extent to which the respiratory quotient became raised as a result of insulin was much less marked than in Page's observations. This may be due to the fact that by the mask method, as used by the latter, the hyperpnœa, which is a prominent symptom of insulin hypoglycæmia in dogs, caused a blowing-off of CO_2, which would affect the results more than when the periods of observation were longer, as in the cabinet.

Measurement of the daily excretion of sugar and nitrogen by

Nash have revealed a decided lowering of nitrogen on the day insulin was given. The D : N ratio also fell and then rose.

Since the mechanism of the action of phlorhizin in producing diabetes is unknown, a study of the effects of insulin on the two chief symptoms does not throw any light on the mechanism of the action of insulin. Both substances partially act on the intermediary metabolic products which stand between sugar and the substances which ultimately are oxidised.

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CHAPTER XVI.

THE INFLUENCE OF INSULIN ON THE RESPIRATORY EXCHANGE.

In depancreatised animals, as also in severely diabetic patients, the respiratory quotient undergoes no change, or, if anything, falls slightly following the ingestion of sugar, but it immediately rises when insulin is also given (p. 123). At the same time, glycogen reappears in the liver (p. 101). The two processes run hand in hand, and it was expected that they would also do so in the normal animal when insulin is injected, more especially when this is done along with sugar, and thus account for the rapid disappearance of sugar from the blood.

There can be no doubt that the sugar which disappears from the blood after the injection of insulin passes into the tissue cells, and here it must either be oxidised, or polymerised, or converted into non-carbohydrate substances. If it is oxidised both the R.Q. and the oxygen consumption must increase. If it is converted, by some reduction process, into non-carbohydrate substances, akin perhaps to the fatty acids, the change will be revealed by an alteration in the respiratory quotient, such as occurs, for example, in hibernating animals while they are feeding richly on carbohydrates and laying on fat in preparation for the winter sleep. Under these conditions the respiratory quotient rises often to well over unity, thus showing that the carbohydrate, in getting rid of its intramolecular oxygen, is giving off CO_2. An analogous process must also occur when the change does not proceed to completion, but only so far as to produce some of the intermediary bodies between carbohydrates and fats. Such a change in R.Q. might or might not be accompanied by a change in the absolute amount of oxygen retained in the body. Another possibility is that the combustion of carbohydrate might be increased, but that of protein and fat correspondingly diminished, the change involved being merely an

alteration in the proportion of the proximate principles involved in the metabolic process. In such a case the quotient might rise without there being any increase in oxygen consumption. If this shift were one taking place between carbohydrate and protein, rather than between carbohydrate and fat, evidence of its occurrence would be obtained by examination of the nitrogen balance.

The first respiratory observations on normal animals were made in November, 1922, by Dickson and Pember, on a dog weighing 7 kg., and in these it was found that the respiratory quotient rose from 0·86 before insulin to 1·16 in ninety minutes after it. The total energy expenditure also rose from 15·35 to 25·5 calories per hour. In numerous other observations of the same nature, on this and another normal dog, it was found that the extent of the increase in calorie expenditure, and also, usually, in respiratory volume ran approximately parallel with the severity of the hypoglycæmic symptoms, the highest values being, as a rule, attained in two to two and a half hours after injecting the insulin, and lasting for five to five and a half hours, after which they began to fall again. Out of a total of ten observations, made on these two dogs (partly by using a respiratory cabinet and partly by using a mask connected through valves with a counterpoised spirometer), the respiratory quotient was found to rise decidedly in eight, and slightly in the two remaining cases, as a result of the injection of insulin. The symptoms were more marked and the calorie expenditure greater when the animals had been starved for twenty-four hours previous to the observations, than when they had been well fed with carbohydrate. The rise in R.Q. also occurred considerably earlier than the increase in calorie expenditure, in the majority of the observations.

In order to obtain more precise information of the exact relationship of the behaviour of the blood sugar to the symptoms and to the changes in respiratory metabolism, the investigations were repeated, in collaboration with G. S. Eadie, on two other dogs, with results exemplified in curve form in Fig. 24. In the first observations, the dose of insulin was sufficient to cause convulsions in about four hours. During the initial fall in blood sugar, which occupied about one hour, the R.Q. and the O_2 consumption rose only slightly, but by the time some recovery

in the blood sugar had set in—about two hours—the latter had increased very decidedly, accompanied by increased respiration and pulse rate. These changes became very pronounced as the blood sugar, following a transient partial recovery, continued slowly to fall to below the convulsive level, the R.Q. meanwhile becoming lower and lower. In another similar experiment in which the dose of insulin was insufficient to cause convulsions, the blood sugar fell rapidly, so as to reach 0·05 per cent. in thirty minutes, and then, more gradually, so that it was 0·04 per cent.

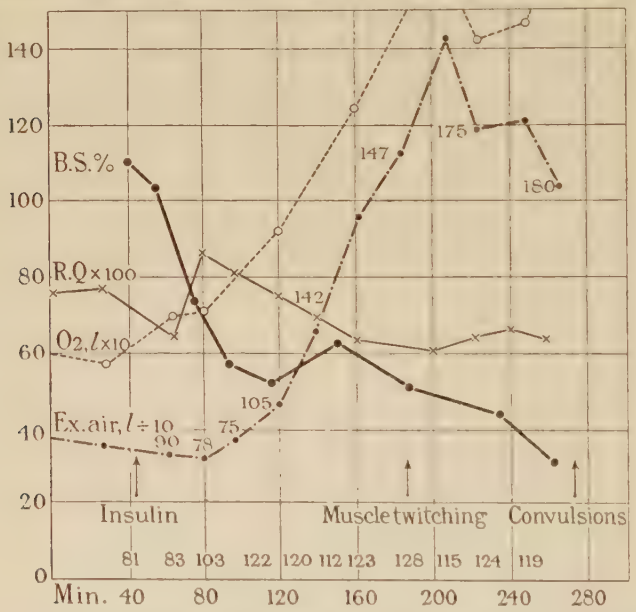

FIG. 24.—Curves showing effects of insulin on blood-sugar, R.Q., O_2 consumption pulse rate and respiration.

in two hours. The R.Q. in this case rose to about unity, while the blood sugar was falling rapidly, but the O_2 consumption did not change until after the initial fall had been completed, when it began to rise and continued to do so steadily for as long as the blood sugar remained low, to fall again shortly after this began to recover. The respiratory volume, the rate of breathing, and the pulse increased parallel with the O_2 consumption.

The results of these and of several other similar experiments clearly show that the respiratory exchange becomes stimulated in the dog when the blood sugar is brought down by insulin to

between 0·045 and 0·060 per cent., and that this is accompanied by violent stimulation of the respiratory centre, and by cardiac acceleration. Although these changes set in before there was any evidence of increased muscular action, they became much more pronounced when this appeared. The energy expenditure usually increased by about 50 per cent., but in one of the observations the increase was much greater, namely, from 28·8 to 60·9 calories per hour. Such an increased metabolism might be expected to entail a rise in body temperature. On the contrary, however, this was invariably found to fall (see p. 278), rising

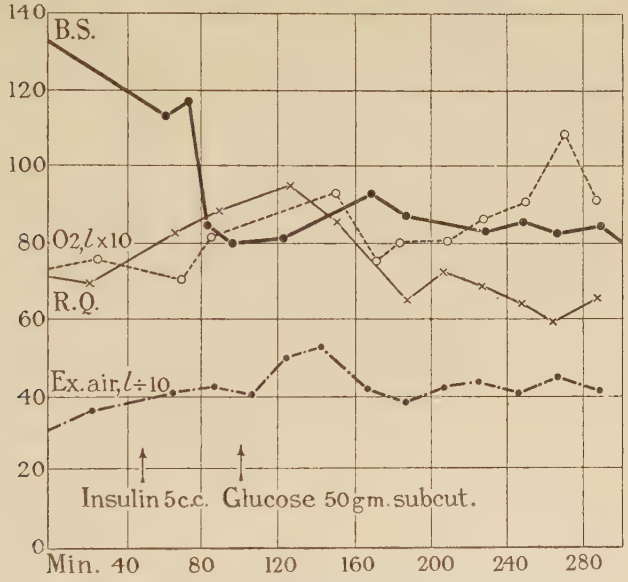

FIG. 25.—Curves showing antidoting effects of glucose on the respiratory changes caused by insulin alone.

slightly following each convulsive seizure. The extent of this fall in dogs was much less than that observed in mice and rabbits (p. 276), not exceeding 2° C., even after massive doses of insulin had been injected.

The outstanding result of these experiments, namely, the increase in respiratory exchange, is undoubtedly related to the lowering of the blood sugar, for it can be entirely annulled by the injection of sufficient glucose to hold this well above the level at which any symptoms supervene. This is shown in the curve of Fig. 25, in the experiment of which 5 gms. of glucose were

injected subcutaneously about one hour after insulin, when the blood sugar had reached about 0·080 per cent., between which and 0·090 it then oscillated for several hours, unaccompanied by any significant increase in O_2 consumption, or pulse rate, and without the appearance of any symptoms. Prior to the injection of glucose, the R.Q. rose sharply nearly to unity, but it fell again after the injection of sugar.

A very constant feature of practically all the observations has been the definite increase in R.Q. occurring about the time of the initial fall in blood sugar. This may depend either on a temporary blowing-off of CO_2 from the blood, as a result of the appearance of acid substances in it, or on primary stimulation of the respiratory centre, or on a change in the type of metabolism to one in which relatively more carbohydrate is undergoing oxidation. Dickson and Pember measured, by van Slyke's method, the total CO_2-combining power of the blood plasma and the total gas ($CO_2 + O_2$) of the blood at various stages following administration of insulin, but they did not find either to be significantly lowered, except at a very early stage, when a slight depression was usually evident. We are uncertain whether this is related to the increase in R.Q., which also occurs at this stage, and whether it indicates that acid substances become developed in the blood at an early stage of insulin action. The H-ion concentration of the blood has also been measured, by the gas chain method, by Brugsch, and no change observed to occur unless convulsions supervened, when it was found to be decidedly increased (pH lowered). Occurring so late, this increased acidity of the blood cannot play a rôle in the increase in R.Q., which occurs about the time the blood sugar is falling rapidly.

Respiratory observations were also made on rabbits, since in them the hypoglycæmic symptoms differ in character from those observed in the dog, in that no period of marked hyperpnœa and muscular twitching precedes the onset of convulsions (p. 276). The animals were observed usually by the mask method, and the results of four experiments are collected together in the curves of Fig. 26.[1] In two cases the O_2 consumption remained practically constant, but it fell slightly in the other two.

[1] The curve for the blood sugar of Expt. I. is omitted.

During the period of the initial fall in blood sugar, the R.Q. rose distinctly in three of the animals, but fell again shortly before the onset of convulsive symptoms. This confirms the observations on dogs, and indicates that some change in the type of metabolism sets in during the period of the initial fall in blood sugar, and continues during the earlier stages of the more gradual decline which follows.

The conclusion which was drawn from these observations

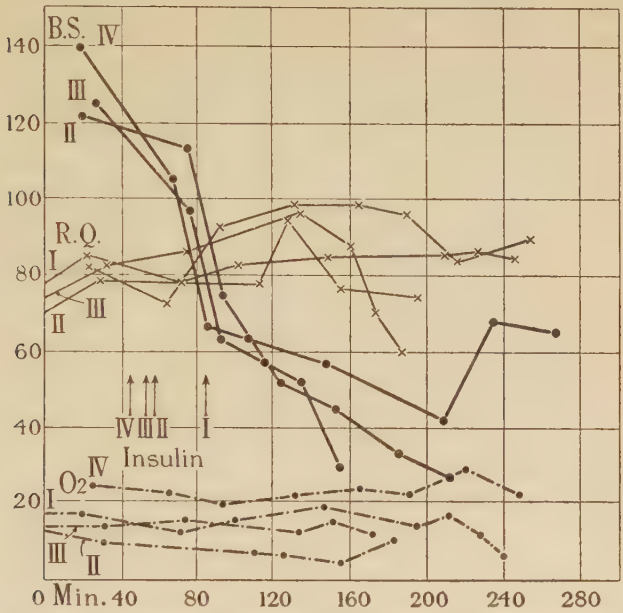

FIG. 26.—Curves showing the behaviour of R.Q. and O_2, consumption in relationship to changes in blood sugar in rabbits injected with insulin.

was that the relative amount of carbohydrate undergoing combustion in the body becomes somewhat greater during the early stages of insulin action, when the blood sugar is rapidly descending, but that this is insufficient to account for more than a small fraction of the sugar which disappears. It was considered that the results, taken in conjunction with those obtained on the behaviour of glycogen (p. 163), indicate that the greater bulk of the sugar which disappears becomes converted into some intermediary form of carbohydrate, which is neither a reducing sugar nor glycogen.

Numerous other investigations of a similar type have been

made on various species of animals in other laboratories, and of these the most important are as follows. Dudley, Laidlaw, Trevan, and Boock (1923) measured either the production of carbon dioxide, or the intake of oxygen in starving mice and rabbits following the administration of insulin, and found both to be depressed. Placing the mice in an incubator at about 30° C., so as to prevent the fall in body temperature, which is a conspicuous feature of the action of insulin in these animals, did not affect the results on the respiratory exchange, except to hasten their development. Administration of glucose retarded the fall, but only temporarily prevented it when not given until after the fall had already set in. Convulsions, when they occurred, did not cause the oxygen consumption to increase. Kellaway and Hughes (1923) observed the respiratory exchange, in the post-absorptive state, of a normal woman of twenty-six years of age. Following the injection of 10 units of insulin, the most significant results were as follows :—

Time.	C.c. O_2 per Min.	R.Q.	Calories per Hr.	Blood Sugar per cent.	
10.00 A.M.	230·6	0·756	66·7	0·125	—
11.30 ,,	224·0	0·741	63·5	—	—
12.30 P.M.	—	—	—	—	10 u. insulin
1.45 ,,	220·5	0·923	65·5	0·080	—
2.45 ,,	229·0	0·860	67·0	0·083	—
3.30 ,,	233·0	0·806	64·2	0·080	—

In another similar observation the oxygen consumption increased from a normal of 213-222 c.c. to 239 c.c. per minute, and R.Q. rose from 0·77 to 0·86. The chief feature of these observations is the very decided increase in R.Q., dependent mainly on an increased CO_2 production. This occurred early in the fall of blood sugar, and therefore affords evidence that a larger proportion of carbohydrate was being metabolised. Based on these results, Dale (1923) suggested that the essential feature of the action of insulin might be to increase the relative proportion of carbohydrate undergoing combustion, without affecting the total respiratory exchange. The authors themselves tried to show, by calculations, that some substance poor in oxygen must have been formed out of the sugar which disappeared. Lyman, Nicholls, and McCann (1923) made observations on five

normal men, and found that insulin caused the respiratory quotient to become raised, usually reaching a maximum in about thirty minutes after the injection (intravenous), although the rise might not set in until a considerable decrease in blood sugar had occurred. The calorie expenditure also became increased, from 2·5–17·7 per cent., reaching a maximum in from ten to sixty minutes, and returning to the basal level in from one and a half to two and a half hours after the injection. It is of interest that, in parallel observations on seven diabetic subjects, the alterations in respiratory exchange were of about the same magnitude, ranging from 2·9–19·6 per cent. It was also found that insulin and epinephrin (which in itself raises the energy metabolism) were antagonistic to a certain extent, since administration of the two hormones simultaneously, or following each other, produced less change in basal metabolism and R.Q. than when either was administered alone. Krogh (1923) determined the respiratory exchange in curarised rabbits, and found, when insulin was injected, that R.Q. increased (especially when it was low to start with), accompanied by no change, or only a slight fall, in O_2 consumption. Although the blood sugar fell promptly following the injection of insulin, the change in respiratory quotient was not manifest until after half an hour. Krogh agrees with Dale's view, that a qualitative change in metabolism, resulting in the oxidation of a higher proportion of carbohydrate, is the essential feature of the action of insulin.

Boothby and Rowntree investigated, more especially, the effect of insulin on the energy expenditure. In resting normal persons in the post-absorptive state no effect was evident, but after starvation an increase occurred when the blood sugar had fallen to a certain critical level. Since these and other investigators have also shown that epinephrin has a stimulating influence on the energy expenditure, the conclusion is drawn that a hypersecretion of this hormone is responsible for the effect following insulin. A similar conclusion has been arrived at by Cannon, MacIver, and Bliss, and both groups of workers believe that an increased secretion of epinephrin is a part of the mechanism by which recovery of blood sugar is brought about.

Noyons, Bouckaert, and Sierens (1924), by using the differential calorimeter of Noyons, have observed, in rabbits, the heat output and the rectal temperature at frequent intervals after

the injection of insulin. The advantage of this method of observation is that the heat output can be determined at frequent intervals. The rectal temperature was found to fall steadily from the time of injection, and the heat output, either to increase or to remain constant until the onset of hypoglycæmic symptoms, after which it started to fall, with occasional remissions, the rectal temperature being at about 34° C. Death occurred when a temperature of 24° C. was reached. Recovery, on the other hand, whether spontaneous or due to administration of sugar, was accompanied by a rise in both heat output and temperature, the one often preceding the other. The suggestion of the authors, that the convulsions of the hypoglycæmic symptom-complex represent the compensatory reaction of the thermo-regulatory mechanism to the lowered temperature, implies that convulsions should not occur when the temperature is prevented from falling, which is contrary to fact (p. 256).

Bouckaert and Stricker (1924) studied the energy output during the simultaneous injection of glucose and insulin in rabbits. When 4 gms. glucose per hour was injected, along with sufficient insulin so that the blood sugar fell from 0·091-0·070 in two hours, the energy expenditure only rose from 0·093 to 0·097 C. per minute after one hour, and to 0·103 C. after two hours. This result indicates that increased combustion cannot be an important factor in accounting for the disappearance of sugar.

In diabetic patients, Noyons and Stricker found the calorie output depressed in one-half hour after the injection of insulin, but it had begun to rise again after one hour.

Among the most important contributions in this field are those of Burn and Dale (loc. cit.), who measured the respiratory exchange in the decapitated, eviscerated preparations described elsewhere (p. 312). During the continuous infusion of sugar without insulin, the O_2 consumption was found to vary in the same direction as the percentage of blood sugar, being, for example, 51-62 c.c. for each five minutes when this was above 0·42 per cent. and 40 c.c., in the same period, when it was 0·240 per cent. When the blood sugar declined, as a result of injection of insulin, a marked increase occurred in the O_2 intake; thus, in the most striking case in one experiment it increased from about 60 c.c. per five minutes to 106 c.c. per five minutes. This

increase set in immediately the insulin was injected, and reached its maximum in ten to fifteen minutes, after which it gradually declined. Although in most of the experiments the increased O_2 consumption was definite, and occurred when the blood sugar was falling, it was far from sufficient to account for all the sugar which disappeared as a result of insulin. Burn and Dale calculated the total amount of sugar which disappeared (by the method outlined on p. 301), and obtained the following results in two experiments :—

Before Insulin.			After Insulin.		
O_2 (Obs.).	Dextrose. Consumed per. hr.	$O_2^{\bar{}}$ (Calc.) Required to Oxidise Dextrose. Consumed.	O_2 (Obs.).	Dextrose Consumed per hr.	O_2 (Calc.) Required to Oxidise Dextrose Consumed.
	(mg.)			(m.g.)	
315	214	160	386	603	450
364	293	218	413	1736	1295

In three other experiments the production of CO_2 was measured at the same time as the O_2 intake. Before injecting insulin the R.Q. stood at about unity (1·02-1·06 and 0·99 respectively), and it remained practically at the same level after its injection (0·92, 0·99, and 1·01 respectively). As others have found (Porges and Salomon), the respiratory exchange of hepatectomised preparations is always about unity, indicating that in the absence of this viscus the muscles can burn carbohydrate only, a fact which indicates, as we have seen elsewhere, that the muscles are restricted to the use of carbohydrate, fats and proteins being unavailable until after they have been transformed into this, or some related substance. The failure of the R.Q. to increase following insulin in the eviscerated preparation shows that the sugar which meanwhile disappears cannot be reduced to fat or to lactic acid, for if either of these changes had occurred quotients of over unity would have been inevitable. These results do not, however, rule out the possibility that a certain amount of the sugar which disappears in a *normal animal*, as a result of insulin, may not depend on formation of fat-like substances in the liver. It is of great interest that similar observations made on cats two days following removal of the pancreas gave results

of the same nature as in normal (eviscerated) animals, thus showing, not only that removal of this gland does not alter the ability of the muscles to use glucose, but also that insulin affects sugar assimilation in the same manner after pancreatectomy as before it.

Other investigations, too numerous to consider in detail here, have, in general, confirmed the results of those already referred to. There can be no doubt that the R.Q. becomes raised soon after the injection of insulin in normal animals. Tsubura (1914) found it to be so, accompanied by an increase in O_2 consumption when hypoglycæmia was induced by insulin, but not when this was prevented by the simultaneous injection of sugar, particularly fructose. When hypoglycæmia became marked the consumption of O_2 became much less, which may indicate a conversion of carbohydrate to fat. Lesser (1924) observed the effect on starved mice of intraperitoneal injections of glucose with or without insulin. The R.Q. rose immediately from 0·77 to 0·83 with insulin and sugar, but did not do so in the first hour with glucose alone, although it rose in the second hour. Both with sugar alone and with sugar plus insulin the intake of oxygen declined somewhat up to the second hour, this decline becoming pronounced when large doses of insulin were given. Lesser considers that the increase in R.Q. indicates increased combustion of carbohydrate, although not of sufficient extent to account for the glucose which disappears. Tolstoi, Loebel, Levine, and Richardson (1924) also found R.Q. to become raised as hypoglycæmia develops, and they believe that this indicates increased combustion of carbohydrates. Heymans and Matton (1924) could detect no increase in the excretion of CO_2 in well fed rabbits after insulin, unless when sufficient was given to induce hypoglycæmic symptoms. When starved animals were used the CO_2 excretion and respiratory volume fell parallel with the decline in body temperature. When glucose and insulin were given together there was no change in the excretion of CO_2, or in respiratory volume. Thus, when 10 gms. glucose was given by continuous intravenous infusion over a period of two to three hours, the blood sugar held at about 0·5 per cent., and the excretion of CO_2 increased somewhat. Injection of large amounts of insulin (60 u.) did not affect the CO_2 output under these conditions, even although it reduced the blood sugar. The

authors conclude that the action of insulin cannot depend on increased combustion of carbohydrate.

Laufberger (1924) concluded that the respiratory exchange is not significantly affected by insulin, although the R.Q. is regularly increased somewhat. Laroche, Grey, and Taquet (1924) found that even small doses of insulin cause R.Q. to rise, both in normal and diabetic men, without any change in O_2 consumption, or in pulmonary ventilation. In rats fed in various ways, Gabbe (1924) observed that the R.Q. became raised, accompanied by a fall in O_2 consumption during a period of about two hours following the injection of amounts of insulin too small to induce hypoglycæmia; the quotient fell and the O_2 intake rose in from three to five hours after the insulin. This worker also observed (cf. Grevenstuk and Laqueur) that flesh-feeding in the case of young rats prevents the fall in O_2 consumption which usually follows injection of insulin, similar effects being obtained by subcutaneous injection of glycin, alanin, creatinin, and guanidin. The rise in R.Q. following the ingestion of carbohydrate was lessened by these substances. Abderhalden and Wertheimer (1924) have confirmed these observations to the extent that insulin was found to excite increased respiratory exchange more markedly in rats kept on a diet poor in carbohydrates than in those fed on one rich in these substances (see also p. 290).

Hawley and Murlin (1924) state that the depression of metabolism, usually observed after insulin, is due to some impurity, since it can be prevented by using highly purified preparations. Although R.Q. still becomes raised after these pure preparations, they believe that this change is not directly related to the fall in blood sugar. Bornstein and Holm (1924) could detect no change in R.Q. but a slight depression in CO_2 output and O_2 intake following the administration to a normal man of an amount of insulin sufficient to lower the blood sugar from 0·088 per cent. to 0·068 per cent. From this and other observations in which glucose was also given, they conclude that there is no evidence that insulin stimulates the combustion of carbohydrate, and they attribute the high quotients often obtained to a washing out of CO_2 from the blood.

Observations on Diabetic Animals.—The effect of insulin on the R.Q. of depancreatised dogs has been described elsewhere

(p. 63), and only brief reference will be made here to other investigations on such animals and on diabetic patients.

Wilder, Boothby, and others (1922) observed that when glucose and insulin were given together the respiratory quotient rose less and more slowly than with fructose and insulin, and the basal metabolism was little changed. Joslin, Gray, and Root (1922), using smaller doses of insulin than the Rochester observers, found, on patients who were on mixed diets, an average increase in basal metabolism of 9·4 per cent., and usually a slight increase in R.Q. In one case the quotient rose to 1·02 as a result of successive doses of insulin. Fitz, Murphy, and Grant (1922) report decided increase in R.Q., lasting sometimes till the third day following the giving of insulin, but unaccompanied, as a rule, by any decided increase in basal metabolism. The persistence of the effect on R.Q. suggests that insulin may have caused storage of carbohydrate in the body, a power much weakened in diabetes. McCann, Hannon, and Dodd (1923), as well as Lyman, Nicholls, and McCann (loc. cit.), have also reported a rise in R.Q. following insulin and glucose. Davies, Lambie, Lyon, Meakins, and Robson (1923) made the interesting observation that the R.Q. may sometimes fall following insulin administration, as was the case in a patient in severe acidosis. They suggest that this result may be due to the fact that the insulin causes disappearance of the ketone bodies, and so releases alkali which, by combining with CO_2, diminishes the relative amount of this gas in the expired air. Rabinowitch (1923), in a comprehensive study of the sugar, the ketone bodies, and the alkaline reserve of the blood, the titratable acid and ammonia of the urine, and the respiratory exchange, points out that the rise in R.Q. which is observed to occur as a result of insulin may be partly due to the combustion of the ketone bodies. Apart from this, however, the quotient, observed under basal conditions of metabolism, also increases as a result of insulin treatment in diabetes, indicating probably increased storage of carbohydrate in the body. This interesting result also shows that there must be considerable persistence of the insulin effect, unless it be that stored carbohydrate can be utilised without the assistance of insulin, or at least requires less of it than is the case with ordinary glucose.

Burgess, Campbell, Osman, Payne, and Poulton (1923) have

also obtained evidence of considerable storage of carbohydrate as a result of insulin treatment in diabetes. In a severe case respiratory quotients of over unity were observed for three successive days, while the patient was receiving very large doses of insulin (75 u. daily).

Bornstein and Kurt (1924) found that insulin improves the power to oxidise carbohydrate, although they do not consider that the increased combustion is adequate to account for the sugar which disappears, the action of insulin being directed to sugar formation in the body, rather than to its utilisation. Incidentally, they state that the CO_2 combining power of the blood is not increased by insulin. In a later paper, Bornstein and Griesbach (1924) attribute the failure of the diabetic organism to oxidise carbohydrate to the absence of glycogen. They also determined the oxygen disappearing from blood while circulating through the muscles, after the injection of insulin, and found it insufficient to account for more than between 20 and 25 per cent. of the sugar which meanwhile disappeared (also determined by comparison of arterial and venous blood).

E. and L. Hédon (1923) found that the O_2 consumption fell in a depancreatised dog when the glycosuria was removed by injection of insulin.

Bernhardt, etc. (1924), observed a rise in R.Q. with little or no fall in O_2 consumption when insulin was given to a diabetic patient in the post-absorptive condition. Lublin (cf. Grevenstuk and Laqueur) emphasises the importance, not only of the restlessness of the patient, but also of the amount of glycogen stored in the body as factors accounting for variability in the results obtained in respiratory observations on diabetic patients.

Taking the clinical evidence as a whole, it is apparent that in diabetes the power to oxidise carbohydrate is decidedly raised by insulin, this effect being often more marked when fructose, rather than glucose, is fed. With this often goes hand in hand a rise in the calorie expenditure. These changes may persist for several days, so that the quotient and the energy metabolism in the post-absorptive state are both higher than before insulin treatment was started.

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CHAPTER XVII.

HYPOGLYCÆMIA.

It is only recently that any attention has been paid to the physiological disturbances which result from a lowering of blood sugar, the opposite condition of an increase, because of its association with diabetes, being considered of much greater significance. The first real contribution to the subject was that of Mann and Magath (1921), who were able to demonstrate that characteristic, and fatal, symptoms supervene when the hypoglycæmia becomes pronounced, their work in this connection furnishing the clue to the interpretation of the supposedly toxic symptoms which follow the injection of excess of insulin. Several other substances, when administered parenterally, can also cause a lowering of blood sugar, but this is not usually of sufficient degree to be associated with symptoms.

Removal of the Liver.—

Bock and Hoffmann (1874), and later Pavy and Siau (1903), found that the blood sugar steadily falls to a low level after removal of the liver from the circulation in dogs. These results were confirmed by various investigators, not alone for animals in which the only operation was removal of the liver (Macleod, 1909; Macleod and Pearce, 1913), but also for animals that had been depancreatised (Chauveau and Kaufmann, 1893; Kaufmann, 1896; Montuori, 1896; Macleod and Pearce, 1913), or asphyxiated (Seegen, 1890; Macleod and Pearce, 1913). For the removal of the liver two methods have, in general, been employed: (1) Joining the portal vein to the central end of the inferior vena cava, by using Crile's cannula and then ligating the hepatic arteries; (2) removing the abdominal viscera, after ligation of the cæliac axis and the anastomotic vessels from the inferior hæmorrhoidal and œsophageal arteries, the portal vein, the hepatic artery and the renal vessels. By the second of these general methods the preparation may really be considered as consisting of heart, lungs and muscles, and it is undoubtedly more suitable for the investigation of the rate of sugar consumption by the muscular tissues than is the heart-lung preparation used by Knowlton and Starling (1912), or Maclean and Smedley (1912). It is advisable in such observations to

obviate the possible depressing effect of anæsthetics on tissue glycolysis, by using decerebrated or decapitated animals. This has been done by Macleod and Pearce in dogs, and more recently by Dale and Burn (1924) in cats, the use of these smaller animals being now possible because of the introduction of the micro methods of blood sugar analysis.

In decerebrate, eviscerated dogs the difficulty which is experienced in maintaining the blood pressure can be circumvented, partly by continuous injections of epinephrin (which has been shown not seriously to interfere with tissue glycolysis) and partly by injecting into the eviscerated preparations defibrinated blood (diluted with Locke's solution) which has been removed from the animal prior to tying off the abdominal vessels. This procedure is adopted in order that as small a quantity of blood as possible may be left in the tied-off splanchnic vessels. In the experiments of Macleod and Pearce the following results were obtained :—

	Mg. Glucose Disappeared from 100 gm. Blood per Min.
1. When no special precautions were taken to maintain blood pressure	0·83-2·4
2. When defibrinated blood was injected	0·46-0·97
3. When epinephrin was injected	1·13-2·8 *

* In one case with epinephrin the rate of disappearance over a short period of time (fifteen minutes) worked out at 4·46 mg. per minute and, in light of this result, and also the general average of the others obtained in this group, it is probable that epinephrin does slightly accelerate the rate of tissue glycolysis, possibly because the blood pressure is better maintained.

There are therefore considerable variations in the rate of tissue glycolysis, but it was impossible to correlate these with the blood pressure, with the presence of anæsthetic or with the injection of epinephrin. It is evident that the rate of disappearance, variable though it may be, is very much greater than that occurring in blood alone (p. 194).

In Burn and Dale's experiments, in which decapitated and eviscerated cats were used, primarily to investigate the effect of insulin, the blood sugar, prior to the injections of insulin, fell in one case from 0·176 to 0·156 per cent. in forty-two to forty-three minutes (about 0·5 mg. per minute). These workers also studied the rate of disappearance when glucose was continuously injected, at the rate of 15-60 mg. in Locke's solution in ten minutes, and they calculated that a total of 410 mg. glucose disappeared in one hour, assuming the combined volume of

blood and of the fluid in the lymph vessels and tissue spaces to equal 500 c.c. for a 3 kg. animal.

Hepatic Hypoglycæmia.—Although it is possible to show, in anæsthetised animals, that the blood sugar rapidly declines after removal of the liver from the circulation, the experimental conditions do not permit of an adequate study of the effects thereby produced on the well-being of the animal. This is partly because they are acute experiments, in which the animal does not recover from the effects of the anæsthetic, and partly because the liver, being merely tied off from the circulation, but left *in situ*, may still have an effect on the systemic blood, due to the ebb and flow of blood through the vena cava and hepatic veins, a process which is assisted by the respiratory movements. With this blood, constituents of the dying liver cells may become washed into the circulation, and these may include not only sugar but also various products of the autolytic process.

To avoid these sources of confusion, Mann and Magath (1921, 1922) have recently elaborated a method in which the liver is extirpated after a collateral circulation has become established between the vena cava and the veins of the thorax.

For this purpose a preliminary operation is performed, which consists of making a reverse Eck fistula by uniting, through lateral anastomosis, the portal vein and vena cava, and then ligating the latter vein central to the anastomosis. The venous blood of the posterior part of the body must then return to the heart through the liver, and the resistance offered to its flow leads, in a few days, to the opening up of collateral channels, through dilation of the azygos and internal mammary veins. In two weeks or so, when this collateral circulation has become established, the portal vein is ligated near where it enters the liver, thus diverting all the blood from the viscera and hind limbs through the collateral vessels. At the third operation the entire liver is removed without any disturbance of the general circulation, so that the animal recovers immediately the anæsthetic is removed, and remains apparently normal for a period varying from two to ten hours.

Characteristic symptoms then supervene, the first being muscular weakness, which quickly becomes more and more marked, so that in a short time the animal is unable to move any of its muscles, except those of respiration. It passes into a state of unconsciousness with the muscles flaccid, and reflex action gone. This condition lasts for a variable period, usually not more than one hour, when the reflexes return in exaggerated

form, accompanied by muscular twitchings restricted at first to separate muscles, but soon spreading until general convulsions supervene, from which death ultimately results. After the symptoms have appeared most animals, if untreated, survive for about two hours. The blood sugar at the appearance of the first symptoms is usually between 0·06 and 0·05 per cent., and as the symptoms develop the percentage gradually falls until, at the time of death, it has reached to between 0·04 and 0·03.

The association of the hypoglycæmia with the incidence of symptoms would not, in itself, warrant the conclusion that the two are causally related, but that this is so, is evidenced by the fact that if glucose is injected at any stage after the development of the symptoms—even, indeed, when the animal is practically dead, and has ceased to breathe—complete and immediate recovery follows. For example, within one minute of the injection of 5 gms. glucose per kg. body weight, a practically moribund animal was restored to the same condition as it was in before any symptoms appeared. The animal may then remain in this tolerably normal condition for an hour or so, when the symptoms reappear associated, as in the first attack, with a decline in blood sugar. A second injection will usually again restore the animal, and this can often be repeated many times with success. After twelve or fourteen hours, however, symptoms of another type become evident, and from these the animal does not recover following the injection of glucose. It dies with the blood sugar at, or above, the normal level. If the glucose be administered slowly after the hepatectomy, as by giving it orally or by rectum, no hypoglycæmia may develop, so that the first set of symptoms do not appear, and, by this method, Mann and Magath have kept an animal alive for thirty-four hours after hepatectomy.

The specificity of glucose in effecting recovery from the earlier symptoms is very striking, although maltose and mannose have also a decided antidoting effect, and to a less extent so also have galactose and dextrin. Other substances of a carbohydrate nature, or related to it biochemically, have no effect, such, for example, as lactose, fructose, and saccharose, of the sugars; and glycerol, glycocoll, lactic acid, and pyruvic acid, of the carbohydrate decomposition products; epinephrin and pituitary extracts are also without action.

It is significant that the hypoglycæmia, after removal of the liver, appeared to develop more rapidly in depancreatised than in normal animals, as was also observed to be the case by Macleod and Pearce. The hypoglycæmic symptoms in depancreatised animals, however, developed at a higher blood sugar level, and although injection of glucose removed them, the beneficial action was not so long maintained.

Hepatectomy in geese, in which the operation is simpler, on account of the natural anastomosis existing in these animals between the systemic and the portal circulation, has given, in

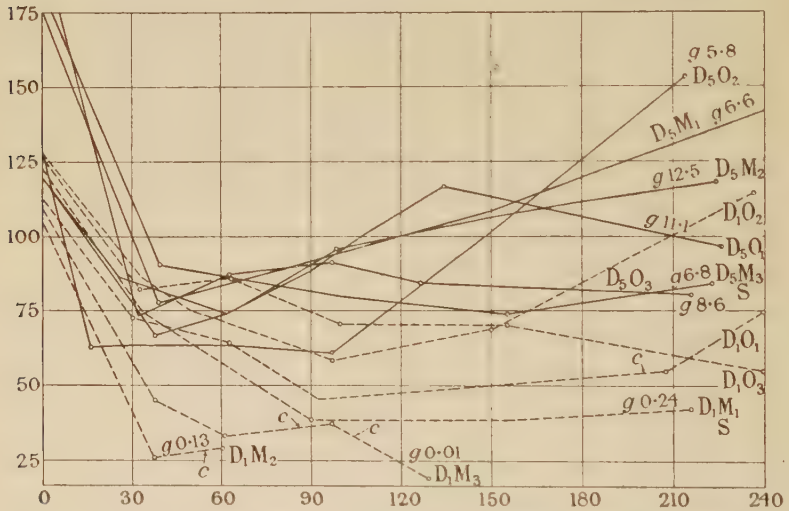


FIG. 27.—Curves showing effect of equal doses of insulin on well-fed (continuous lines) and hungering rabbits (dotted lines). The percentages of glycogen found present in the livers of the various animals are indicated by the figures (*g*) on the curves, (*c*) convulsions. (McCormick, Noble, etc.)

general, the same results as in dogs, the symptoms being, however, less marked, although the fall in blood sugar is relatively as great.

Insulin Hypoglycæmia.—Typical results illustrating the effect of equal doses of insulin on the blood sugar of rabbits are shown in Fig. 27. In these curves the continuous lines represent the results obtained on animals previously well fed with carbohydrate-rich food, and the broken lines those on animals that had been starved for several days and injected either with epinephrin or phlorhizin, so as to render them, as far as possible, free of glycogen (p. 140). The blood sugar, in both groups of animals,

comes down very promptly after injection, and then reaches a low level, at which it may either remain tolerably constant for a considerable period, or commence to rise again. This depends very largely on the nutritional condition of the animals, the significant factor being the percentage of glycogen in the liver, which is indicated by the figures on the various curves, although possibly other factors, such as the acid base equilibrium of the body, may also have some influence. We shall first of all discuss the two phases of the curves in their relationship to the glycogen content of the liver.

Initial Fall.—The fall in blood sugar sets in practically immediately after the injection of insulin, and there is remarkably little difference in the time of onset, according to whether this is made intravenously or subcutaneously. The fall occurs steadily for about forty-five to sixty minutes, and its steepness is practically independent of the amount of glycogen in the liver, provided the blood sugar of the animal, before the injection, is at the usual level. When, as is sometimes the case in well-fed animals, the blood sugar to start with is higher than usual, it descends more rapidly following the injection of insulin, and, although it may not attain so low a level, the absolute extent of the reduction is greater. Comparing the results following intravenous and subcutaneous injections, the fall in blood sugar in one-half hour is usually greater in the former, but after an hour there is practically no difference between the effects.

The initial fall in blood sugar is, within wide limits, independent of the dose of insulin administered, and it is only when the doses are very minute that any grading of the intensity of the reaction can be shown to exist at this stage. Such a relationship can be seen in a general way in the accompanying curves (Fig. 28), which represent results in which varying small quantities of the same insulin were injected into four rabbits, all as nearly alike as possible in weight, age, and nutritional condition. In one hour after the injection a certain relationship between dosage and effect can be made out when, as is the case in the curves on the left side of the chart, the results actually obtained are replotted to the same normal value of blood sugar to start with. The application of these results in the assay of insulin will be referred to later (p. 342). In the curves shown in the right-hand part of Fig. 28, such correlation, however, is not evident.

Recovery Process.—In well-fed animals the blood sugar, after an average dose of insulin, usually regains the normal level in three to five hours, but in those that have been deglycogenated recovery, if it occurs at all, may be much delayed beyond this period. The rate of recovery varies, therefore, very greatly with both groups of animals, even although the dose of insulin may be exactly the same. This is clearly seen in the curves of Fig. 27, in the experiments of which the same doses of insulin were injected intravenously. As a rule, the rate of recovery is much more uniform in animals that have been partially deglycogenated by moderate starvation, than in those in which there are large stores of glycogen in the liver.

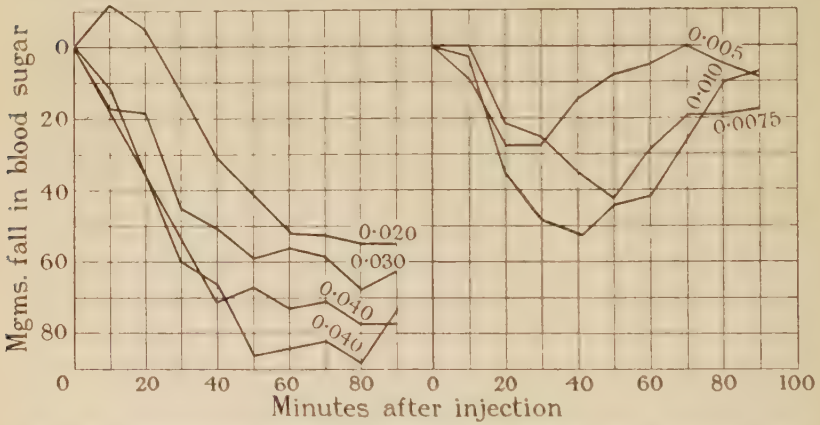


FIG. 28.—Curves showing the fall in blood sugar during the hour following the injection of varying doses of insulin as indicated by the figures standing on the curves. (Macleod and Orr.)

These relationships indicate clearly that the recovery of blood sugar is dependent on secretion into the blood of sugar derived from the glycogen stores of the liver, and two questions present themselves—first, the cause for the stimulation of the glycogenolytic process; and, second, the cause for the delayed recovery which occurs when little or no glycogen is available. The glycogenolytic stimulus does not apparently become developed until the blood sugar has reached a certain low level, otherwise the initial fall would be slower in glycogen-rich than in deglycogenated animals. That the stimulus, whatever it may be, acts on the liver stores of glycogen, is evidenced by the fact, first demonstrated by Burn and confirmed by Markowitz,

that recovery of the blood sugar is greatly delayed in well-fed rabbits after paralysis of the sympathetic nervous system by ergotamin. Burn and Marks have found the same effect to follow section of the splanchnic nerves. McIver, Cannon, etc., concluded that onset of stimulation of increased hepatic glycogenolysis coincides with a hypersecretion of epinephrin, since they have found, after removal of the medulla of the adrenal glands in cats, that a small temporary increase in blood sugar, which in normal animals is often observed as the first step in the recovery process, is not evident.

When a certain threshold of blood sugar is reached stimulation of the glycogenolytic process occurs, and there must be two factors, at least, involved in determining the outcome of this stimulation. One of these is the inherent sensitivity of the glycogenolytic mechanism in the liver, and the other, the available amount of stored glycogen. These are not interdependent processes, for there may be abundant glycogen which is so firmly bound in the liver cell that a much stronger stimulus is required to mobilise it than when only a trace is present in a loose state. In other words, the percentage of glycogen found present in the liver does not necessarily indicate whether or not mobilisation of sugar is likely to occur readily. When only traces are present the sugar output cannot last for long without a process of gluconeogenosis coming into play, and there is some evidence to show that such occurs after insulin, although it is a slower process than that of glycogenolysis. It accounts for the recovery in blood sugar which occurs, even in animals with no trace of glycogen in the liver.

The recent work of Burn and Marks (p. 338), in which the response to insulin, of rabbits, has been shown to be greatly affected by administration of thyroxin, and of Bodansky and Burns and Marks, showing that thyroidectomy increases the sensitiveness of the animal to that hormone, when taken in conjunction with the work of Cramer (p. 337), indicates a most important influence of the thyroid over the glycogenic function.

Behaviour of the Blood Sugar Curve, following Insulin, in Other Animals than the Rabbit.—

(1) *Dog.*—The typical effect of insulin on the blood sugar of this animal, as shown in Fig. 24 (p. 252), is very similar to that

obtained in rabbits, with the difference that it is not so steep, the minimum being reached in about two hours. The curve then rises somewhat, but this is not, as a rule, long continued, being followed by a further decline, which continues so that the lowest level is reached in from four to five hours. This temporary slight rise in the curve, following the completion of the initial fall, is more frequent in dogs and cats than in rabbits. In the cat the fall in blood sugar proceeds much as in the dog, the initial fall being completed in about one and a half hours, and the lowest level reached, often after a temporary rise, in about three hours.

(2) *Man.*—Fletcher and Campbell (1922) have compiled, in curve form, the results obtained from five normal, and fifteen diabetic subjects, insulin being given in the morning before breakfast after a sample of blood had been taken for normal sugar. Further samples of blood were taken, at intervals of about one hour each, for a period of about four hours following the injection. No very evident difference could be made out between the effects observed in diabetic and normal subjects, but there was a tendency for the extent of the fall to be dependent on the normal blood sugar level, the higher the blood sugar, the greater the fall. In two of the observations in which the blood sugar was observed for longer than four hours, there was slight recovery in one; in the other, the curve continued to fall for another hour, after which it remained steady. It may be concluded that, in man, the sudden initial fall, so characteristic of smaller animals, particularly the rabbit, is replaced by one which is much more gradual, the difference being probably dependent on the relative rates of the circulation time. It was noted that the extent of fall in blood sugar did not bear any accurate relationship to the amount of insulin given. In one patient, for example, 20 units brought about a greater fall than 30 units, and almost as great as 50 units.

Cold-blooded Animals.—The first observations in this connection were made by Noble and Macleod (1923), who were unable to detect any lowering of blood sugar in five to six hours following the injection of insulin in turtles. Observations were not then made on frogs, on account of the very low amount of sugar normally present in the blood of these animals, at least when they are kept under laboratory conditions. Meanwhile, Krogh

informed me that Rehberg had observed that insulin has a delayed effect on frogs, as judged by the development of the characteristic symptoms of hypoglycæmia (see p. 281). His collaborator, Hemmingsen, later published observations in which a decided decrease in blood sugar occurred in twenty-four hours after injecting insulin into two frogs. In butterfly larvæ, on the other hand, an increase occurred, and no effect could be observed in the rate at which injected sugar disappears from the blood, either of the larvæ or of crayfish, as a result of insulin. It was later observed by Olmsted (1924) that the blood sugar rises considerably when the frogs are kept for two days at a temperature of 22° C., but on this hyperglycæmia he did not find insulin to have any effect. McCormick and Macleod (1925) studied the effect of insulin on *Myoxocephalus* (sculpin), but it was impossible to demonstrate that it can cause the normally low blood sugar to become further lowered. In certain of these experiments the insulin was given daily for some time before the fish was killed for the collection of blood; in others it was injected only for a period of a few hours preceding the blood examination. The lowest percentages obtained were 16 and 8 mgs., which might occur in uninjected animals (p. 198).

Houssay, Sordelli, and Mazzocco (1923), and later Houssay and Rietti (1924), have also published accounts of numerous investigations on the effects of insulin on different cold-blooded animals, including small crocodiles, frogs, toads, turtles, snakes, and fish. In crocodiles (*Caimen sclerops*) the injection of 10-60 u. insulin caused decided hypoglycæmia in twenty-four hours, after which it became more marked until, on the third or fourth day typical symptoms supervened. Subsequently the blood sugar rose somewhat, but the symptoms recurred at intervals, and the animals died in from seven to ten days after the injection. In Argentine frogs (*Leptodactylus ocellatus*), and toads (*Bufo marinus*), hypoglycæmia was evident in five to eight hours after injection of insulin, and in one to four days characteristic symptoms were observed.

Mann, Bollmann, and Magath found that intravenous injection of large quantities of insulin in Rana pipiens, and also in the fish (*Lepisosteus plastostomus*), was followed by hypoglycæmia after twenty-four to thirty-six hours, the lowest level of blood sugar being reached in sixty to ninty-six hours.

These uncertain results on the blood sugar of cold-blooded animals are of considerable interest, since, as we shall see later, symptoms simulating those due to hypoglycæmia in warm-

blooded animals have been observed to become developed several days after the injection of insulin (p. 281).

Hypoglycæmic Symptoms.—In the hypoglycæmia due to insulin, as in that following hepatectomy, definite symptoms appear when the blood sugar falls to a certain level. These are known as the hypoglycæmic symptoms. They vary somewhat in different animals.

(1) *Rabbit.*—Premonitory symptoms of hyperexcitability, increasing timidity, and evidence of extreme hunger may be observed before the first definite symptoms appear. These usually consist of violent convulsive seizures, in which the animal throws itself over sideways, first in one direction, then in the other, with the head retracted and the hind limbs in an extended position. Sometimes they consist of extreme drowsiness, gradually increasing to coma. The convulsions are not unlike those caused by strychnine or by acute asphyxia, except that they are more restricted to certain groups of muscles. After a period, which varies from thirty seconds to a minute or so, the convulsions cease and the animal lies on its side apparently in an unconscious state, with rapid, shallow breathing, and dilated pupils. The exact condition of the animal at this stage may, however, vary considerably, well-fed and, therefore, glycogen-rich animals, often behaving in apparently normal fashion between the convulsive seizures, which may be brought on by attempts to move, and are more violent than in those that have been starved. Animals that have been frequently injected with insulin sometimes appear to acquire a certain degree of immunity to the convulsions. After a varying period the coma is followed by another convulsive seizure, and these phases may continue alternately for an hour or more, the convulsions becoming feebler and feebler, until at last the animal dies of respiratory failure. As the coma becomes more developed the rectal temperature falls. The relationship of these symptoms to the blood sugar is shown in the following protocol :—

Experiment, 24th April, 1922.

8.10 a.m.—Blood sugar, 0·129 per cent. ; 5 c.c. insulin given subtaneously.

8.55 a.m.—Blood sugar, 0·077 per cent.
Animal in convulsions some time before 11.30.

11.40 a.m.—Blood sugar, 0·047 per cent. ; rectal temperature, 37·1° C.

11.48 a.m.—2·5 c.c. more insulin injected.
12.00 noon.—Convulsions, rectal temperature, 36·0° C.
12.10 p.m.—Blood sugar, 0·033 per cent. ; rectal temperature, 36·0° C.
12.15 p.m.—Convulsions.
12.18 p.m.—5 gms. dextrose in 40 c.c. water injected in several places subcutaneously ; rectal temperature, 36·0° C.
12.23 p.m.—Blood sugar, 0·056 per cent. ; rectal temperature, 36·5° C. ; rabbit now sitting up and apparently normal.
12.43 p.m.—Blood sugar, 0·091 per cent. ; rectal temperature, 36·5° C. ; rabbit normal.
1.05 p.m.—Blood sugar, 0·070 per cent. ; rectal temperature, 37·6° C. ; rabbit normal.
2.35 p.m.—Blood sugar, 0·043 per cent. ; rectal temperature, 38·0° C.
3 35 p.m.—Blood sugar, 0·053 ; rectal temperature, 37·6° C. Convulsions.
5.40 p.m.—Blood sugar, 0·024 ; rectal temperature, 35·0° C. Convulsions.

Although definite convulsions usually appear at about 45 mgs. per cent. in animals that have been fed, they may not do so until the blood sugar is much lower in starved animals, particularly when a large dose of insulin is injected. The reason for these differences is probably dependent on the fact that it is the concentration, or rather the tension, of glucose in the nerve cells that is responsible for the symptoms, and this will run much more closely parallel with the blood sugar when the latter does not change quickly, than when the changes are sudden. In well-fed animals, for example, the tendency to the development of hypoglycæmia will be partially counteracted, after the initial fall in blood sugar, by the mobilisation of sugar from the glycogen reserves, and will descend to the convulsive level at such a rate that there is time for complete diffusion to take place between the blood and the tissue fluids. In starved animals, on the other hand, the blood sugar, after the initial fall, continues its rapid descent to below the level at which symptoms may appear, because time has not been allowed for this diffusion to occur. Out of a total of 971 animals starved twenty-four hours and injected with insulin, no symptoms were observed in 158, although one or more of the blood sugars was decidedly below 0·040 per cent. (Macleod and Orr, 1924). Besides these factors, it must also be remembered that certain rabbits are more susceptible to convulsions as the result of hypoglycæmia than others. Indeed, there is some evidence that occasionally an

animal may develop a certain refractoriness towards insulin, by its repeated injection (see p. 276).

(2) *Dog.*—In the dog the first signs are usually very rapid breathing, restlessness, and general hypersensitivity, coupled with loss of interest in its surroundings and indifference to the attention of its master. Muscular twitching then becomes evident, and the sphincters may relax. Barking is often a prominent symptom, and there may be salivation and frothing at the mouth. At this stage, or it may be as the first symptom, convulsions, not unlike those seen in the rabbit, may supervene, and between them the animal lies on its side evidently unconscious, showing violent twitchings of the musculature, amounting almost to a tetany. The rates of breathing and of the pulse increase. Inspiration is usually short and jerky, and inspiratory tetanus not infrequent, so that artificial respiration may have to be applied. Attempts to get on its feet are often the cause for convulsive seizures, and during recovery the muscles of the extremities, particularly the anterior, are seen to have entirely lost their power of co-ordinate action. In etherised dogs even massive doses of insulin have no immediate effect on the blood pressure. The rectal temperature falls as the hypoglycæmia develops, but it rises decidedly following convulsive seizures. This is illustrated in the following protocol :—

Dog, 8 kg. (16th July, 1923).

10.50 a.m.—Blood sugar, 0·098 per cent.
10.55 a.m.—Rectal temperature, 38·65° C.
11.0 a.m.—Large dose of insulin.
12.20 p.m.—Blood sugar, 0·067 per cent.
12.25 p.m.—Temperature, 38° C.
12.55 p.m.—Animal obviously ill.
1.50 p.m.—Animal appears to be recovering. Given more insulin.
2.42 p.m.—Blood sugar 0·048 per cent. Temperature, 37·3° C.
3.35 p.m.—Temperature, 36·9° C.
3.45 p.m.—Temperature, 37·0° C.
3.46 p.m.—Convulsions.
3.48 p.m.—Temperature, 37·4° C.
4.15 p.m.—Temperature, 37·3° C.
4.30 p.m. Given cane sugar by stomach tube.

(3) *Cat.*—The behaviour of this animal is well described in the following protocol from Olmsted and Logan's paper (1923) :—

Weight, 2·6 kg.

12.30 p.m.—0·8 c.c. insulin injected subcutaneously.

3.0 p.m.—The cat is becomng weak and comatose ; lies on its side and is hard to rouse ; emits a peculiar cry ; has tremors in hind limbs ; pupils of eyes dilate and constrict in rapid succession ; defecates and urinates.

3.10 p.m.—Cat has become hypersensitive ; twitching of both front and hind limbs ; responds to auditory stimuli such as whistling by opening its eyes wide, pupils becoming greatly dilated ; knee jerks elicitable on the slightest touch ; gives flexion reflex when toes are pinched ; respirations rapid and shallow ; salivating freely ; defecates.

Appears uneasy, raising its head. Blood sugar at this time was 0·046 per cent.

3.20 p.m.—Respirations very rapid and shallow ; muscular twitching all over the body ; head is thrown back ; pupils widely dilated ; front legs rigid and extended, hind legs limp ; makes walking movements with front legs ; rolled over to right once or twice ; cries continuously, salivating freely ; whirling does not produce nystagmus. Blood sugar, 0·043 per cent.

3.25 p.m.—Respirations extremely rapid and shallow ; cries ; head thrown far back ; front legs rigid, hind legs limp ; tail shows some rigidity ; makes walking movements with front limbs ; rolls over and over to the right, emitting a peculiar cry all the time ; the hair, particularly that along the back, stands up straight. Blood sugar, 0·036 per cent.

3.30 p.m.—Ten cubic centimetres saturated dextrose solution injected subcutaneously.

3.55 p.m.—Front limbs appear normal ; hind limbs are weak and show some inco-ordination ; can sit up, but sways slightly from side to side.

3.58 p.m.—Six cubic centimetres saturated dextrose solution injected subcutaneously. Animal fairly normal though weak.

9.0 p.m.—The animal was found in convulsions again. Ten cubic centimeters of saturated dextrose solution and 1 c.c. epinephrin injected subcutaneously.

9.0 a.m.—The animal has apparently entirely recovered, except that it walks slower than normally.

(4) *Mouse.*—In these small animals the symptoms vary somewhat in detail, coma being usually a more prominent symptom than convulsions. This is particularly so if the animals are kept at room temperature, since under these conditions the body temperature very quickly falls, as was first observed by Krogh. When the animals are placed in an incubator at a temperature between 25° to 30° C., convulsive symptoms are more

prominent, and their incidence, as we shall see later, may be used for purposes of pharmacological assay. They usually develop well within an hour after the injection of insulin, although their onset may be delayed until two hours.

Restlessness and irritability are first observed, and at this stage a tap on the box, or a sudden noise, is likely to make the animal jump. The tail then stiffens and may be held arching over the back. These preliminary symptoms are soon followed by convulsions, the most common form of which is that the mouse springs into the air and then on landing, runs about in an inco-ordinate fashion, often falling over sideways. Sometimes, however, the convulsive seizures consist rather of a tonic spasm effecting the whole musculature and lasting for a few seconds. In the intervals between the convulsions or spasms, the animal usually lies sprawling in a toneless condition, with the legs extended, this position being very characteristic in the mouse. Convulsive seizures can usually be brought on by handling the animal. The convulsive symptoms gradually become less marked and the mouse looses all power of movement, passing into a state of extreme collapse and coma, and making no attempt to right itself when placed on its back. Respiration also becomes very slow and protrusion of the eye-balls and dilatation of the pupils are common symptoms. If the dose of insulin has not been too large, spontaneous recovery may occur, and if this be from a state of coma, convulsive seizures, gradually becoming stronger, may reappear during the recovery. It is remarkable from what a deep degree of coma an animal may recover, a fact which must be borne in mind in investigating the effect of various substances on the symptoms.

(5) *Man.*—The following is the description given by Fletcher and Campbell (1922) :—

"The initial symptom may be a feeling of nervousness or tremulousness, sometimes a feeling of excessive hunger, at other times a feeling of weakness or a sense of 'goneness.' The level at which a patient becomes aware of the fall in blood sugar is usually fairly constant for that individual. When a reaction has already been experienced, the onset of a subsequent one is usually recognised by the patient when the blood sugar percentage falls to some point between 0·08 per cent. and 0·07. Usually this is rapidly followed by objective signs—most frequently a sweat which may be very profuse ; pallor and flushing is common ; sometimes a change in pulse rate. In children this increased pulse rate is often the means of detecting hypoglycæmia ; in adults, the sweat is the outstanding feature. At the same time the subjective symptoms become more severe ; the feeling of nervousness may become definite anxiety, excitement, or even emotional upset. The feeling of tremulousness is possibly a form of inco-ordination. Patients have shown a loss of power to perform fine movements with their fingers.

Actual tremor has not been observed at this stage. At times there is a feeling of heat or cold, sometimes of faintness. Some have complained of vertigo ; others of diplopia. This is the extent of most reactions, and the blood sugar is usually between 0·07 per cent. and 0·05 per cent. Much more severe manifestations are observed with further lowering of the blood sugar. Marked excitement, emotional instability, sensory and motor aphasia, dysarthria, delirium, disorientation, confusion, have all been seen, but not convulsions." With the greater possibility of detecting the onset of symptoms in man, because those of a subjective nature can be observed, the degree of hypoglycæmia at which they first make their appearance is much less than in laboratory animals. This level varies, however, in different individuals. " There are patients who become aware of the hypoglycæmia when the blood sugar is between 0·08 per cent. and 0·09 per cent. On the other hand, other patients have experienced no symptoms at levels as low as 0·054 per cent. A severe reaction has been observed with a blood sugar percentage ot 0·060 per cent., and a determination of 0·04 per cent. has been obtained during the course of a mild reaction. A blood sugar percentage of 0·035 per cent. is usually accompanied by unconsciousness. The lowest blood sugar observed was 0·025 per cent."

(6) *Symptoms in Cold-blooded Animals.*—We observed that when insulin was injected into frogs no symptoms of hypoglycæmia became developed, the animals being kept under observation for four days at room temperature. A few months later, A. Krogh informed us that symptoms did supervene when the frogs were kept after injection for longer periods, and that these symptoms were comparable with those induced in mammals.

The symptoms consist of hyper-excitability followed by muscular inco-ordination, so that the animal fails to maintain its equilibrium, sometimes there are convulsive seizures. Olmsted (loc. cit.) subsequently found that the onset of the symptoms could be greatly accelerated by warming the injected frogs. He observed that " immediately before the convulsion the skin of the frogs becomes lighter in colour, the males usually croak, the limbs becomes rigidly extended as in strychnine poisoning, the eyes are covered by the nictitating membrane, and the pupils are dilated. Suddenly the frog leaps about most vigorously, falling backward and often rolling over or turning back somersaults. This vigorous convulsion lasts only a few seconds. The frog now becomes limp, respiratory and other muscular activity ceases. Often the lungs remain fully inflated so that the sides of the frog are puffed out and the animal floats, nostrils under water and legs hanging limp. Within a few minutes it lifts its nostrils out of water, opens its eyes, flexes its legs, and begins to breathe very rapidly. Usually a second convulsion does not take place for an hour or so, when the entire performance is repeated." For reasons already mentioned, it was

found impossible, however, to correlate the incidence of these symptoms with the blood sugar level. In observations by Julian Huxley and Fulton (1924), it was found that it took from five to six days for the symptoms to appear when the animals were kept at 7° C., whereas it took only twenty-four to twenty-seven hours in the case of those kept at 25° C. These observers noted that the time of incidence of the symptoms did not bear any relationship to the dose of insulin, within wide limits. They also found that the symptoms developed quickly when frogs that had been kept cool for several days after injection were warmed.

In the summer of 1923, Olmsted continued his studies on the effect of insulin on cold-blooded animals, using the fresh-water catfish (*Amerius nebulosus*), and he describes, in fish kept after injection for two days at room temperature, peculiar symptoms, which he considers to be related to hypoglycæmia.

"The melanophores of the fish at the time of injection of insulin were in the contracted condition, but on the following day they had expanded fully and the fish became jet black and remained so for four or five days. A few hours before the actual convulsion the fish appeared weak and unable to swim against a gentle current of water. At this time also it was practically insensitive to stimuli, such as touch, etc. While thus being passively carried around the tank, suddenly it would dart through the water at a very rapid pace, often gaining such momentum that it was carried out over the edge of the tank. Then occurred a period of inability to maintain its equilibrium. If it was quiet at this time it would roll over very slowly to one side, righting itself with a quick jerk, or the tail would rise slowly until the fish stood on its head, then suddenly the tail would be brought down with a jerk or the fish would start swimming. The sudden dashes through the water soon took a spiral course, and the fish progressed in corkscrew fashion. The rotation noted in some twenty fish was, in general, to the animal's right.

A few individuals were seen turning to the left, and one turned over straight backwards. When the turning movements ceased the fish either floated motionless, head out of water and tail straight down, or sank to the bottom and lay motionless on its side. The gills ceased to move and the animal was quite inert. After several minutes the eyes began to roll, then the gills to move, the fish now recovered its balance and usually swam along the surface, mouth out of water gulping air vigorously. Frequently, instead of dashing about the vessel in a spiral course, the fish would give a convulsive shudder and move backwards in a series of jerky movements. Immediately after a convulsion the fish became weak again and also refused to respond to stimuli such as touch, vibration, etc. A few hours later, however, it became hypersensitive and dashed about the tank, even when the table was struck at some distance from it." The effect of the insulin was found to wear off gradually if the fish was left at room temperature. At the same

time McCormick observed similar symptoms in other fish, namely, sculpin (*Myoxocephalus*), and Sea raven (*Hemitripterus*).

The development of symptoms in these animals is particularly interesting, because of the fact, which has already been pointed out (see p. 274), that it has been impossible to make certain that any significant fall in blood sugar really occurs after the injection of insulin. On the other hand, since insulin can retard the development of asphyxial hyperglycæmia in the sculpin, it is probable that some reduction of the normal blood sugar may occur (McCormick and Macleod, 1925). It has been common to assume that the above symptoms are hypoglycæmic in nature, but it must be remembered that in many regards they simulate those of lack of oxygen, which, as is well known, is a condition difficult to avoid in sea-water fish kept in aquaria. It is possible, therefore, that asphyxia rather than hypoglycæmia may have been in part responsible for the symptoms observed.

The Cause of the Symptoms.—The symptoms give the impression that some substance having a highly irritative influence on the central nervous system has become developed in the body, and the striking similarity of certain of them to those which can be induced by "spinning" a normal rabbit, suggests that the vestibular apparatus is being stimulated. The most definite point of difference between the two conditions is that nystagmus is absent in insulin convulsions, though it is a conspicuous symptom following spinning; moreover, spinning an animal showing the premonitory symptoms of hypoglycæmia does not bring on a convulsion. There is some evidence that interference with the oxidative processes in the nerve cells may be related to their irritation, although the symptoms still occur in animals kept in an atmosphere rich in oxygen. Olmsted and Logan (1923) have pointed out that the symptoms in the cat and rabbit are, in many regards, like those which may be induced by sudden asphyxia. This lack of O_2 in the nerve cells may be the result, either of a failure in the supply carried to them by the blood, or of the development within their protoplasm of some condition which renders them incapable of utilising the oxygen with which they are supplied. Some support is given to the former possibility by the fact that the arterial blood, when the hypoglycæmic symptoms supervene, has often been noted to be venous in colour.

In view of these facts, Olmsted and Taylor (1924) have measured the degree of oxygen saturation of the arterial blood in decerebrated and decapitated cats, and have been able to detect a slight, but nevertheless definite fall at the period just preceding insulin convulsions. In rabbits, however, no conclusive results could be obtained when the arterial blood was examined, although Argyle Campbell and Dudley found that, following the injection of insulin, the percentage of oxygen becomes much reduced in air injected subcutaneously some time previously. These results are all the more significant when taken along with the fact that in the cat and dog, hyperpnœa is one of the precursory symptoms of the hypoglycæmic condition. There can be no lack of oxygen intake by the lungs, and Olmsted and Taylor's results indicate that there is no breakdown in the transport of oxygen by the hæmoglobin. We are compelled to conclude that the inspired oxygen must be locked away in some form in the tissues, with the consequence that its tension in the blood supplying the brain becomes inadequate, and so causes symptoms of anoxæmia.

With regard to the *locus of action of the toxic factor*, little that is definite is known.

Olmsted and Logan observed that insulin did not cause hypoglycæmia, or symptoms, in decerebrate cats, provided the section of the brain stem was well forward, but did so in typical fashion when it was far enough back so as not to destroy the pituitary gland. Cannon afterwards pointed out that injury to the *Hypothalamus* in the latter operation was the factor responsible for these different results. In any case, the low-decerebrated animals developed typical convulsions when the blood sugar fell sufficiently, whereas other animals, after decapitation, did not show them. These results indicate that excitation of the mid brain, pons, or medulla must be responsible for the symptoms. This would seem to be supported by the observation of Kleitman and Magnus in which it was demonstrated, that after section of the spinal cord convulsions occurred, as a result of insulin, only anterior to the section. But Olmsted and Taylor subsequently found that symptoms extremely like those of insulin hypoglycæmia supervened in a decapitate preparation which was very actively respirated; convulsive seizures of a violent type could be brought on by the feeblest stimulus, until after the injection of glucose, when the symptoms entirely disappeared. It would seem, therefore, that the toxic factor associated with hypoglycæmia may also act on the spinal cord, although its action is usually located on the mid brain centres. Kleitman and Magnus were able to destroy the fore part of the cerebellum and the labyrinth without the susceptibility to

symptoms being lost. These workers also made a careful study of the order in which the nervous disturbances become developed, the progressive reflexes and then those of posture (Korperstell reflexe and Drehnystagmus) being affected, but not those of the turning reactions and compensatory eye movements.

The Relative Value of Various Sugars and Similar Substances in Removing the Hypoglycæmic Symptoms.—Whatever may be the nature of the toxic stimulus, it is clearly related to reduction in the amount of glucose in the blood and tissues (see p. 276). The immediate removal of the hypoglycæmic symptoms which follows the addition of glucose to the blood, either by way of the alimentary tract or parenterally, is sufficient evidence of this. It becomes of interest, therefore, to determine the value as antidotes of other sugars and related susbtances, since by so doing light may be thrown on the physiological significance of the various combining groups that compose the glucose molecule, and of their stereometric relationships to one another. Studies of this nature were first made by Mann and Magath on the hypoglycæmia resulting from removal of the liver in dogs (p. 268). They concluded that mannose, maltose, and galactose, but not fructose, had restorative effects, though of a distinctly inferior order to that of glucose.

Noble and Macleod (1923) made a thorough investigation of the same nature in the case of rabbits showing insulin convulsions and concluded as follows : (1) The sugar which can most definitely antidote the symptoms that accompany the hypoglycæmia due to insulin is glucose. Even when the animal is moribund at the time of injection this sugar may bring about permanent recovery. Mannose is almost, if not quite, as efficacious as glucose. (2) Fructose, galactose, and maltose are often followed by improvement in the symptoms, and they cause a marked increase in the blood sugar. The improvement is, however, usually only temporary, the animal relapsing into symptoms from which it is liable to succumb, unless injected with glucose or mannose. Sometimes with maltose permanent recovery can be effected, although the antidoting action develops more slowly following the injection than is the case with glucose. (3) Arabinose, xylose, sucrose, and lactose have no apparent effect on the symptoms, although there may be an increase in the reducing power of the blood (e.g., with arabinose) ;

(4) sodium lactate, glycerol, and alkalies have no effect on the symptoms.

More recently Herring, Irvine, and Macleod (1924) have extended these observations so as to include a more complete list of sugar derivatives. Most of the observations were made on mice, a few also with maltose on dogs, cats, and rabbits. The substances investigated may be classified as follows :—

1. Unsubstituted Reducing Sugars.

Glucose.—This was used chiefly to establish the standard against which other results might be compared.

Mannose, Fructose, Galactose, Maltose, Lactose.—The use of the above compounds determined the effect of the reducing group in conjunction with different asymmetric systems.

2. Substituted Reducing Sugars.

Tetra-acetylfructose, 2 : 3 : 5 : 6-Tetramethylglucose, 2 : 3 : 5-Trimethylglucose.—The above compounds were employed so as to ascertain the effect of the reducing group alone.

3. Glucosidic Compounds.

Sucrose, α-Methylglucoside, β-Methylglucoside, Tetramethyl-β-methylglucoside, Tetramethyl-γ-methylglucoside, Glucose monoacetone, Salicin.

4. Alcohols Related to the Sugars.

Mannitol, Dulcitol.

5. Anhydro-Sugar.

β-Glucosan.

Positive results, that is, removal of the symptoms, were obtained only by the sugars of the first of these groups and by tetra-acetylfructose. The unsubstituted sugars varied considerably, however, in their antidoting powers, glucose and mannose being of about equal potency, then, although acting more slowly, maltose, and much inferior, since they effected only partial recovery (immediate abolition of irritability and convulsions, but followed by relapse with no permanent recovery), fructose, galactose, and tetra-acetylfructose.

From these results a number of generalisations are justified. In the first place, it is evident that the presence of the reducing group is essential, since entirely negative results were obtained with all non-reducing compounds, and even with α- and β-methylglucosides, in which the reducing group is modified to the least

possible extent. Since *l*-arabinose does not remove the symptoms, it may be concluded that the cyclic structure of the sugar molecule is important (Irvine).

As Irvine has pointed out, certain asymmetric systems of the sugar chain are also necessary. This does not apply to position 2, for whereas in glucose the arrangement is $H-\overset{|}{\underset{|}{C}}-OH$, the reverse arrangement $HO-\overset{|}{\underset{|}{C}}-H$ is present in mannose, and both of these sugars are equally effective as antidotes. It also appears that the primary alcohol group is not involved, since maltose, in which this group is substituted, is effective.[1]

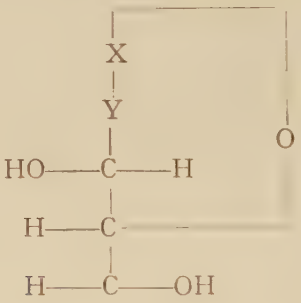
This leaves the carbon atoms numbered 3, 4, and 5 in the glucose chain to be considered. These may be written as follows :—

HO——C——H 3
H——C——O 4
H——C——OH 5

and have the same arrangement in mannose and maltose. Substitution of 5 destroys the curative action, as evidenced by the negative results with lactose and arabinose, and this is of some interest, as pointed out by Irvine, because this position is involved in the ring structure of the molecule of glycogen. Position 4 is that involved in the ring formation of glucose, and when this occurs so that the O_2-atom is on the opposite side of the chain of carbon atoms the positive reaction is diminished, as, for example, in the case of galactose. Whatever may be the exact significance of the grouping of each of these three atoms, it is evident that their asymmetric systems are ineffective in the absence of the reducing group, as is instanced by the negative behaviour of arabinose, xylose, and lactose, these being sugars which have the aldehyde group, but not the complete asymmetric systems. This conclusion is confirmed by the behaviour of tetra and trimethyl glucose, in which methyl radicles mask the hydroxyl groups, leaving the reducing groups free. On the other hand, it is interesting to note that both mannitol and sucrose are negative, although in them the necessary asymmetric system

[1] Although this may depend on its rapid inversion to glucose in the blood.

is present, but the reducing group absent. When a mixture of galactose, which contains a normal reducing group, and methyl glucoside, in which the asymmetric system is present, is injected, no antidoting effect is observed, showing that both of the above conditions must be satisfied in the one molecule. As expressed by Irvine : " A review of the combined results show no exception, which cannot be adequately explained, to the generalisation expressed in the statement that the *type of carbohydrate molecule functional in eliminating the convulsion symptoms occasioned by the administration of insulin is :—*



where either X or Y represents a reducing group." It is of interest that glucose monoacetone is without effect, since this compound has been shown to be a derivative of γ-glucose, into which it is readily hydrolysed by very dilute acids. Irvine has further suggested that two series of reactions are really involved, one of them being an attack on the reducing group of sugar, and the other one in which the asymmetric chain of CHOH groups participates. This opinion is supported by the peculiar behaviour of galactose, which, as it were, carries the antidoting effect half-way.

It is significant that the sugars which act as antidotes for the hypoglycæmic symptoms are also those that are directly fermentable by yeast, namely, glucose, fructose, and mannose. Galactose, which is much less fermentable than any of the other hexoses is, as we have seen, decidedly inferior as an antidote. With regard to the evidence, that the primary alcohol group is not significant, it must be pointed out that this is based on the effects of maltose, and it is possible that the decided results displayed by this disaccharide may be dependent not on the fact that the masking of this group is of no consequence, but rather

because the maltose is immediately hydrolysed to glucose by the maltase, which has been shown by Bial (1893) to be present in blood. It is significant that this sugar is decidedly less effective in antidoting the symptoms in the rabbit, cat, and dog than in the mouse, which may depend on more rapid hydrolysis in this animal. A characteristic feature of the action of maltose in the larger animals is that the improvement in the symptoms which often results shortly after its injection is not continued, relapses being frequent, so that ultimately it may be necessary to inject glucose.

We have already called attention to the fact that the recovery in blood sugar which may slowly occur in insulin-injected rabbits, from which practically all of the liver glycogen had previously been removed, must indicate that sugar is being formed out of protein or fat. This opens the opportunity to determine what substances may serve as precursors of glucose in the animal, for if it is found that the blood sugar recovers quickly after their injection and the symptoms of hypoglycæmia disappear, it may be assumed that glucose (or possibly fructose) has been formed. Noble and I thought that we could in this way make out a certain gluconeogenesis from lactic acid, but more convincing results have been obtained by Voegtlin and Dunn, who used standardised white rats in their experiments. They found, for example, that intraperitoneal injection of glycerol into rats that were almost moribund as a result of insulin hypoglycæmia, was followed by recovery from the symptoms, and Voegtlin has since informed me that this recovery is accompanied by a rise in blood sugar. Of course, there is always the possibility, in such experiments, that the injected substance may excite sugar mobilisation from any traces of glycogen left in the liver, in the same manner as occurs when epinephrin is injected, or even in the entire absence of glycogen, that it may stimulate gluconeogenesis out of protein or fat, but, nevertheless, the experiments are highly suggestive, and their further prosecution should yield important results.

Other Factors Influencing the Response of Animals to Insulin.—The difference in the effects of insulin on well-fed as compared with starved animals has been considered in the present chapter as primarily dependent upon the amount of glycogen stored in the liver. But other dietetic factors may also play a rôle, and among these emphasis has been placed on

the ash content, through its influence on the acid base equilibrium of the body. I. H. Page found that rabbits kept on an acid-forming ration of oats and bread are more resistant towards insulin than those kept on a base-forming one of oats, carrots, and cabbage, the average CO_2 combining power of the blood of animals on the acid-forming diet being 51 per cent., and in that of those kept on base-forming diet, from 74·4–77·4 per cent. Blatherwick and others have confirmed this, and have further shown that the differences are not dependent on the glycogen content of the liver, which, after twenty-four hours' starvation, is the same in both groups of animals.

Abderhalden and Wertheimer have confirmed and extended these observations. In rats starved for some time, insulin causes symptoms less readily than in those fed with carbohydrate before a short period of starvation, and the blood sugar falls more rapidly in animals kept on food rich in carbohydrate than in those on food poor in this regard. On the other hand, epinephrin causes more marked hyperglycæmia in the latter group of animals. That these differences in behaviour are related to differences in the acid base equilibrium was shown, in rabbits, by observing the CO_2 combining power of the blood, the reaction of the urine, and the rate of sinking of the corpuscles on allowing the blood to stand after withdrawal. Tiitso, in my laboratory, has also found that the blood sugar, when measured at short intervals immediately after the injection of insulin, falls much more rapidly in rabbits fed with sugar and carrots than in starved animals. These experiments were undertaken primarily to see the whether recovery of the blood sugar would occur earlier when liver was rich in glycogen than when little glycogen was present.

These investigations indicate clearly that the percentage amount of glycogen in the liver is not the only factor which determines the rate of recovery from insulin hypoglycæmia (see also p. 272). There is evidently something of greater importance than the amount of glycogen, and although this may be related to the acid base equilibrium, it is probably also dependent upon the influence of some other internal secretion acting on the sensitivity of the glycogenolytic mechanism. It is undoubtedly wrong to assume that the readiness with which sugar can be mobilised in the body is determined by the amount of glycogen present in the liver; indeed, it is probable that when

conditions have become established for a building up of this substance, its breakdown can be excited less readily than when there is a tendency to the opposite process, as, for example, when the alkaline reserve is at a low level. Evidence has been gradually accumulating to show that the thyroid glands play an important rôle in the control of the glycogenic mechanism (p. 337), and it is possible that it is through their influence that different feeding conditions affect the action of insulin.

Other Causes of Hypoglycæmia.—Space will not permit of more than a brief reference to the hypoglycæmic effects of other substances than insulin. Some of these, such as phosphorus and the salts of uranium, and of various other heavy metals, produce only slight and doubtful effects on the blood sugar. Thus Bowie and Pember were unable in my laboratory to obtain any constant results with phosphorus given in various ways to rabbits. Certain antipyritics, opium, and atropin are also said to cause a lowering of blood sugar, but the evidence is unsatisfactory. On the other hand, considerable hypoglycæmia may be caused by coccidiosis (Collip, 1923) and by feeding with foodstuffs having a strongly basic ash. Injection of salvarsan and of peptone tends also to lower the blood sugar.

The effects of administration of alkalies, both on pancreatic hyperglycæmia, which was especially investigated by Murlin and his collaborators (p. 60), and on the tolerance of animals for carbohydrate, studied by Elias and others, have also a bearing in this connection. Hydrazin sulphate causes a decided lowering of blood sugar associated with extensive necrotic destruction of the liver cells (Underhill and Karelitz). When large doses are given to dogs the most pronounced degree of hypoglycæmia occurs in forty-eight hours, and is associated with alkalosis (Hendrix and McAmis). In view of the extensive damage to the liver cells caused by phosphorus, hydrazin, and many metallic poisons, it is not surprising that the blood sugar should become reduced in amount.

The most outstanding hypoglycæmic effects, however, are those produced by guanidin and by glucokinin. Quanidin sulphate causes decided hypoglycæmia in rabbits after several hours, and symptoms may supervene which can be at least temporarily relieved by injection of glucose. Thus, Frank and others found that the development of these symptoms is related

to the glycogen stores of the animals, starved rabbits and mice showing symptoms like those due to insulin when the blood sugar reached 0·050 per cent., whereas well-fed animals were immune. Undoubtedly, however, these symptoms are not entirely dependent on hypoglycæmia, since dimethylguanidin can cause them without at the same time affecting the blood sugar. Administration of guanidin to diabetic patients is said to have no effect in reducing the hyperglycæmia (Izar). Its influence on depancreatised dogs has apparently not been investigated.

Glucokinin is the name given by Collip to extracts prepared from yeast, and a variety of plant tissues, by methods similar to those used in the preparation of insulin, and which have the property of causing the blood sugar in normal and diabetic animals to become reduced in amount. But a striking difference exists between the effects of insulin and glucokinin, in that the latter develop slowly and are long maintained. Sometimes, as with the earlier extracts prepared from yeast, the blood sugar increased immediately following the injection, then fell gradually so as to reach the lowest level in from ten to twenty-four hours, when convulsive symptoms supervened at about 0·045 per cent. of blood sugar. Injection of glucose could often relieve the symptoms, although it could not prevent death in many of the injected animals. In order to obtain satisfactory yields of glycokinin, thorough maceration of the tissue, prior to its extraction with hot water, was found to be important, and to ensure this it was first of all frozen with CO_2 snow. The extracts were then purified by fractional precipitation with alcohol. Sometimes the hypoglycæmic effect was not evident until several days after the injection, but since the rabbits were meanwhile starved it is difficult, as Collip himself points out, to be certain that this was really due to the glucokinin. Administered to depancreatised dogs, glucokinin also reduces the hyperglycæmia and diminishes the excretion of sugar by the urine, but it is uncertain whether the animal can be maintained in normal condition, as we have seen is the case when insulin is used. In one such animal given glucokinin prepared from onion tops, the blood sugar was maintained at about the normal level for some days, but, since abscesses developed, it is difficult to determine to what extent the extract was really responsible for the prolonged effect.

A most remarkable outcome of these experiments was

demonstration of the fact that the hypoglycæmia-producing principle can be transmitted from animal to animal to an indefinite extent. When, for example, a few cubic centimetres of defibrinated blood, or serum, from a rabbit in hypoglycæmia was injected into a normal rabbit, the blood sugar became reduced, usually within twenty-four hours, often to a sufficient degree to cause convulsions or death of the animal. Injection of a similar amount of the blood of this animal into a third one had exactly the same effects, and the injection from animal to animal could be repeated with similar results until, in some cases, over twelve such reinjections had been made. A variety of agents may be used to induce the hypoglycæmia in the first animal of the series—glucokinin, overdosage with insulin, guanidine sulphate, infection with coccidia, or prolonged fasting—and it is evident that some very potent hypoglycæmic agent becomes developed in the inoculated animals, since a lethal dose may be contained in as small an amount of passage blood as 0·05 c.c. This material is heat resistant; for example, it is not destroyed by autoclaving at 15 lb. pressure, and it can be concentrated by boiling, it can be dialysed, it is not precipitated with the proteins when tungstic acid is added to blood, although it can be removed from the blood by ammonium sulphate.

The hypoglycæmic symptoms, caused by passage blood, are extreme weakness, convulsions, and death. Injection of glucose can restore the animals only temporarily. Although they may eat voraciously, extreme emaciation occurs, which has led Collip to suggest that extensive breakdown of protein is occurring. Unfortunately there are no data as to the behaviour of the blood sugar in these fed animals, so that, as Collip himself points out, the hypoglycæmia in injected ones may in part be due to the starving condition. It is suggestive also that blood from hepatectomised dogs can be used for injection into the first animal of the series. Whatever this remarkable substance which becomes developed in the blood of the passage animals may prove to be, there is no doubt of the great significance of these excellent observations by Collip. Glucokinin is probably simply some form of compound of insulin with some substance which, perhaps because of a hyperglycæmia-producing action, delays its typical effects, and in this connection it is interesting, as shown by Dubin and Corbitt, that typical insulin can be prepared from

glucokinin by adsorbing the latter on wood charcoal and then acting on the adsorbed compound by glacial acetic acid. Fisher and McKinley, and Collip himself, have confirmed these observations.

Glucokinin has been found by Ellis to have an interesting influence on the growth of seedlings of maize. When it is dialysed, a substance passes through the membrane which retards growth, whereas the residue in the dialyser aceelerates it. In plants treated with concentrated dialysate for several days, drops of brownish exudate with strong reducing properties develop on the tips of the leaves, after which the plants die and show decided changes in the starch granules.

When it was found that insulin-like substances could be prepared from such easily procurable substances as yeast, it was hoped that this might prove a fruitful source of supply for its manufacture. Winter and Smith, who paid much attention to this problem, found that only certain strains of yeast were suitable, and apparently the yields were uncertain. In any case, there is now no call for other sources of raw material for the manufacture of insulin, the yields from mammalian pancreas being entirely adequate, at comparatively small expense, for all possible demands.

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CHAPTER XVIII.

MECHANISM OF THE ACTION OF INSULIN.

The Effect of Insulin on Glycolysis.—The rapidity with which the blood sugar falls following the injection of insulin, suggests that an intravascular reaction of some sort may, in part at least, be the responsible cause. It is true, when one considers the large quantities of glucose which can be caused to disappear from the blood when insulin and glucose are injected together, that increased assimilation by the tissues must be the main factor involved, but, nevertheless, the possibility exists that increased glycolysis in the blood itself may be a contributory factor. Experiments to put this possibility to the test were carried out by Eadie, Macleod, and Noble (1923). Insulin, in varying quantities, was added to sterile, defibrinated dog blood, and the rate of glycolysis was compared with that of a duplicate sample of the same blood not containing insulin, but no difference could be detected between the two.

Since insulin might have to combine with something in the body before displaying its glycolytic powers, the foregoing observation was repeated, with the difference that the rate of glycolysis was compared in blood removed before injecting an animal with insulin with that occurring at intervals after injection. The results of such an experiment are shown in the following table :—

	Time.						
	10.50.	12.00.	1.00.	2.00.	3.00.	4.00.	5.00.
Per cent. glucose A . .	0·094	0·085	0·086	0·032	0·020	Trace.	—
Per cent. glucose B . .	—	0·05 ?	0·073	0·028	0·016	,,	—
Per cent. glucose C . .	—	—	0·046	0·049	0·051	0·058	0·60

in which A gives the rate of disappearance of sugar, at the times indicated, in blood removed before insulin was injected; B the same in blood removed an hour after the injection; and C the sugar in the blood remaining in the animal's body. Insulin was injected at 10.50 and 11.40. From this last value (C), it is evident that insulin of high potency was used, and yet, by comparing the results of A and B, it can be seen that there was no fundamental difference in the *in vitro* rate of glycolysis, the slight difference between A and B observed after one and two hours being probably due to the fact that the temperature of the shed blood was temporarily below that of the body.

The above experiment was repeated, using rabbit blood, with exactly similar results.

Thus, in blood removed from an insulin-treated animal, in a hundred minutes after insulin, the percentage of glycolysis occurring in one hundred and fifty minutes at room temperature was 22 per cent., as compared with 22·4 per cent. in the control blood of a normal animal. Inasmuch as after an interval of this length, any differences due to insulin might have worked themselves out, these observations were repeated with the modification that the blood was examined at much shorter intervals after its removal from the body. In order to do this, the method followed was to remove some blood (3 or 4 c.c.) from the ear vein, then inject insulin and remove some blood about ten minutes later. Both bloods after defibrination were then incubated at body temperature and samples of 0·1 c.c. removed at intervals of a few minutes apart and the sugar determined by the Hagedorn-Jensen method (1923). In one such experiment, in twenty-six to thirty minutes after removal of the blood, 7 per cent. of glucose disappeared from the blood before injection of insulin, and only 5 per cent. after its injection. It is evident, therefore, that increased glycolysis is not responsible for the initial fall in blood sugar.

The negative character of these results led us, step by step, to investigate other possibilities, such as, whether insulin might influence the rate of glycolysis in mixtures of muscle juice and blood, and in suspensions of leucocytes (in sterile pus). In the experiments of the former category the procedure was to compare the rate of disappearance of sugar in well-buffered (phosphate) mixtures containing blood along with either Buchner extract of muscle prepared with sterile precautions or finely chopped muscle, insulin being added to some of the flasks, but not to others. In another group of experiments the comparison was made between mixtures obtained from normal animals and

from those previously injected with insulin (in certain of the experiments, instead of adding insulin, a preparation of pancreas, described by Cohnheim and by Hall (1908), was used). Typical results of these experiments were as follows :—

Experiment.—Rabbit 1 was given 4 c.c. insulin at 10.30 a.m. and killed at twelve noon. The sugar in the defibrinated blood was 63 mgs. per 100 c.c. Normal rabbit 2 was killed at 10.45 a.m., the blood obtained and defibrinated, and muscle juice prepared by Buchner's process. These were kept in the ice-chest until ready. The pancreas of this rabbit (2) was thrown into boiling water, the extract evaporated almost to dryness, alcohol was added, filtered off, evaporated to dryness, and the residue taken up in distilled water (Cohnheim). The following mixtures were made :—

(*a*) 10 c.c. blood from rabbit No. 2 ; 4 c.c. muscle juice ; 4 c.c. usual phosphate mixture ; two drops 10 per cent. dextrose solution.

(*b*) 10 c.c. blood from rabbit No. 2 ; 4 c.c. muscle juice ; 4 c.c. phosphate ; 2 c.c. insulin ; two drops 10 per cent. dextrose solution.

(*c*) 10 c.c. from rabbit No. 2 ; 4 c.c. muscle juice ; 4 c.c. phosphate ; two drops 10 per cent. dextrose solution ; pancreas preparation described above.

(*d*) 10 c.c. blood from rabbit No. 1 ; 2 c.c. muscle juice ; 2 c.c. phosphate ; two drops 10 per cent. dextrose solution.

(*e*) 10 c.c. saline : 2 c.c. muscle juice ; 2 c.c. phosphate ; two drops 10 per cent. dextrose solution.

ANALYSES.

Time.	Glucose per 100 c.c.				
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)
	mgs.	mgs.	mgs.	mgs.	mgs.
12.35 p.m.	180	185	173	129	85
1.35 ,,	140	182	157	111	82
2.35 ,,	160	182	157	113	75
3.35 ,,	137	167	135	99	91
4.35 ,,	137	162	137	77	80
5.35 ,,	119	155	119	62	83
Percentile glycolysis after three hours .	23·8	9·1	22·0	25·2	—

The rate of glycolysis was the same in blood *plus* muscle juice (*a*) as in blood *plus* muscle juice *plus* Cohnheim's extract (*c*). It was, however, decidedly slower when insulin was added (*b*). During the first three hours of incubation glycolysis was practically the same in mixtures of blood and muscle juice (*a*) as in the blood of the insulin-injected animal and muscle juice (*d*).

It can be seen that there is no evidence that insulin accelerates the process; on the contrary, in the experiments instanced, as also in others of the same type, the addition of insulin produced decided retardation of the glycolysis. It is possible that this may have been due to slight differences in pH, although, as already explained, large quantities of buffer were used. In any case, the insulin certainly does not accelerate the process.

With regard to the possible influence of insulin on the rate of disappearance of sugar from sterile pus, equally negative results were obtained.

These observations were made according to the instructions of Levene and Meyer (1912), the pus being obtained from the pleural cavity of a dog into which, some days previously, turpentine had been injected. In one experiment of this type, in which two flasks, containing the same amount of sterile pus and phosphate mixture along with glucose, were incubated side by side, it was found, after eleven hours, that 87·4 per cent. of sugar had disappeared in the flask containing insulin and 86·6 per cent. in the control. Since some of the sugar which disappeared might have been converted into glycogen or some other polysaccharide, the fluids remaining in the flasks were hydrolysed with acid. Although a slight increase occurred in the reducing power, this did not account for more than a small fraction of the sugar which disappeared, and only traces of glycogen could be detected in the incubated fluids. It is concluded that insulin has no effect on the rate at which sugar disappears from sterile pus incubated outside the body. It should be pointed out that in certain details these observations were not conducted in exactly the same manner as those of Levene and Meyer (1912), the differences being that the pus cells were not washed with isotonic saline, as these authors recommend, and that the concentration of glucose in their experiments was much greater than in ours.

Finally, several experiments were undertaken to see if insulin could influence the rate at which glucose disappears when it is fermented by yeast, or by *B. coli communis*. Entirely negative results were obtained. This has been confirmed by Travell and Behre (1924), by Heymans and Matton (1924), Laufberger (1924), von Fürth (1924), Ducceschi (1924), and others.

The Extent to which Insulin can Cause Glucose to Disappear from the Organism.—The mysterious manner in which glucose disappears from the organism following its injection in normal animals has already been alluded to (p. 218). Still larger amounts disappear when the sugar is injected a short time

after insulin. Evidence of this was obtained in the following experiments: (1) Eadie and Macleod, in experiments already referred to (p. 208), found that the percentage of blood sugar, previously lowered by insulin, was scarcely changed when quantities of glucose (2 gms. per kilo body weight) were injected, which in normal rabbits would have caused marked hyperglycæmia. When 2 gms. glucose per kilo body weight were injected into each of three rabbits which had received insulin, a little over an hour previously, the rises in blood sugar were only 22, 39, and 53 mgs. respectively (average 44 mgs.), whereas when injected into normal rabbits this amount of glucose causes the blood sugar to increase from 64 to 205 mgs. (see p. 207); (2) analogous results were obtained on dogs. Thus, Dickson, Eadie, Macleod, and Pember (1924), after giving an animal weighing 12·6 kgs. sufficient insulin to lower the blood sugar to 0·080 per cent., injected 50 c.c. of a 2 per cent. solution of glucose subcutaneously without any change occurring in the blood sugar level, and, it may be added, without any change in oxygen consumption.

(3) Burn and Dale (1924) determined the rate at which glucose was removed from the circulating fluid of decapitated and eviscerated cats (see p. 258), and found, in one animal weighing 2·6 kgs., that this was 410 mgs. per hour before injecting insulin, and 914 mgs. per hour after doing so. In this experiment considerable hypoglycæmia resulted from the injection of insulin. In other experiments the tendency to hypoglycæmia was counteracted by increasing the rate of infusion of glucose, and under these conditions the disappearance of glucose became enormous. Thus, during a period of forty minutes before insulin, the rate of disappearance of sugar in mgs. per hour was 369, whereas during four consecutive twenty-minute periods following it, the figures were 1704, 1026, 1392, 1452 respectively.

(4) Bissinger, Lesser, and Zipf (1923) have investigated the problem by measuring, at regular intervals, the increase in the amounts of free sugar and of glycogen produced by the injection into white mice of 0·050 gms. glucose (0·25 per cent. of body weight), either with or without insulin. In mice starved eighteen hours the free sugar was found to amount to 22 (± 1·1) mgs., and the glycogen to 20 (± 1·6) mgs. After the injection of glucose the following values were obtained :—

	Time After Injection.	Sugar.		Glycogen.	
		Total Found.	Amount Disappeared.	Found.	Amount Deposited.
		Mg.	Mg.	Mg.	Mg.
A.	½ hour	56	16	25	5
Without	1½ hours	45	27	35	10
insulin	2-3 „	26	46	42	22
B.					
With	½ hour	29	43	36	16
insulin	1 „	22	50	20	0

Insulin greatly increased the rate at which the free sugar disappeared from the entire animal and in 30 minutes three times as much glycogen was deposited in the liver as was the case in the controls. In one hour, however, the glycogen was found to correspond to that obtained in starved mice. It would appear from these results that the increased disappearance of glucose is linked with more rapid glycogen formation, but that the glycogen thus formed does not remain (see also p. 163).

These results clearly show that the disappearance of sugar from the blood following insulin cannot depend merely on an inhibitory action of this hormone on the process of sugar formation in the liver or elsewhere in the body. If such were the mechanism involved, the rate at which the hypoglycæmia becomes developed following insulin should correspond to that which occurs after hepatectomy, and administration of insulin to animals in this condition should not alter the contour of the blood sugar curve. Mann and Magath (1923) have, however, shown, not only that hypoglycæmia develops after insulin much more rapidly than after hepatectomy, but also that when insulin is given to animals in this condition the blood sugar curve behaves exactly as in a normal animal. These results do not necessarily prove that the liver may not participate with other organs and tissues in locking away some of the sugar that disappears, but they would seem to rule out the possibility that the primary cause is one resident in this viscus.

It is clear that the immediate cause for the hypoglycæmia is increased diffusion of sugar from the blood into the tissues, because of the development in them of a lowered tension of glucose. There can be no doubt that the glucose which passes

from the blood into the tissues, exists in the latter partly in simple solution, creating, as it were, a certain tension of glucose, upon the magnitude of which, in relationship to that of the blood, will depend the rate of diffusion of blood sugar into them. Besides being in equilibrium with the blood sugar, this tension of glucose in the tissues must also be in a certain equilibrium with intermediary products of sugar metabolism, so that a disturbance at any stage in the chain of processes will extend all the way along it. Insulin must act by exciting some stage of sugar metabolism beyond free sugar, so that a vacuum for sugar becomes created in the tissues—a condition of *glucatonia*, we may call it—and, as a consequence, glucose is removed from the blood. Valuable evidence that an equilibrium exists between the tension of glucose in the tissues and the blood sugar is afforded by the observations of Folin and Berglund (p. 217), and also by the fact that glucose is present in the aqueous humour (Starkenstein, 1911), and in the cerebro-spinal fluid in concentrations not far removed from that in blood.

Two leading problems now present themselves for consideration: (1) To what extent are the various organs and tissues involved in the glucatonic action of insulin? (2) what becomes of the sugar that disappears? We shall consider the former of these in the present chapter, reserving for other ones (XII. and XVI.), an examination of the evidence as to whether the sugar is converted into glycogen or is completely oxidised.

The Extent to which Different Organs and Tissues are Involved in the Hypoglycæmic Action of Insulin.—There are, in general, several methods by which this problem may be attacked, and of these the most important are: (1) By comparison of the sugar concentration in the blood flowing into and out of an organ before and after injecting insulin. This is done in samples of blood removed from the appropriate vessels in an intact animal; (2) by observing the rate at which sugar disappears from the nutrient fluid perfused through an organ outside the body; (3) by finding whether the effect of insulin is modified or altered by removal of one or more organs from the animal.

The Effect of Insulin on the Relative Sugar Concentrations in the Blood Flowing into and out of an Organ *in situ*.—It is essential in this method, not only that the two samples of blood be removed simulatenously, but also that numerous successive

samples be taken, so as to make certain that the small differences in the percentage of sugar which at best can be expected to exist, because of the large volume flow of blood, are not due to experimental error. In cases where the differences are small, it is also important that possible changes in the volume flow and in the water content of the blood be controlled. This method has been used in investigating the action of insulin by Hepburn, Latchford, McCormick, and Macleod (1924), by Cori, Cori and Goltz (1923), Foster, Lawrence (1924), Frank, Nothmann and Wagner (1923), Wertheimer (1923), and Faber (1923), with results which are not entirely in harmony.

In our observations etherised dogs were employed, and blood was removed, practically simultaneously, from the portal vein, the femoral artery, the femoral vein, and, in some observations, from the vena cava opposite where the hepatic veins join it. In nine experiments the average difference between the arterial and venous blood of the muscles (femoral artery and vein) before insulin was injected was 0·016 per cent. (minimum, 6, and maximum, 26), and that between the inflowing and outflowing blood of the liver 0·009 per cent. (minimum 0, maximum 17). At varying periods after the injection of insulin, in four experiments, the results shown in Table XXI. were obtained.

TABLE XXI.

Sugar per 100 c.c. Blood.					Remarks.
Femoral Artery.	Femoral Vein.	Difference F.A.–F.V.	Portal Vein.	Difference F.A.–P.V.	
Mg.	Mg.	Mg.	Mg.	Mg.	
94	83	11	94	0	Before insulin.
80	70	10	74	6	10 min. after insulin.
70	55	15	58	12	*45 ,, ,,
179	159	20	162	17	Before insulin.
150	129	21	134	16	35 min. after. insulin.
145	140	5	107	38	55 ,, ,,
162	148	14	160	2	Before insulin.
138	126	12	140	+2	36 min. after insulin.
135	107	28	117	18	48 ,, ,,
155	138	17	145	10	*100 ,, ,,
103	96	7	99	4	Before insulin.
91	88	3	78	13	During injection.
61	83	+	74	+	*18 min. after injection.

* Arterial blood pressure very low (30 mm. Hg.).

The relative sugar values of the inflowing and outflowing bloods did not become significantly altered as a result of insulin, although the blood sugar was falling.

Since the range through which the blood sugar can fall, in an animal in which this is at the normal level, is a limited one, it was attempted to exaggerate the differences between inflowing and outflowing blood by causing hyperglycæmia, either exogenously, by the continuous injection of glucose, or endogenously, by injecting epinephrin or by asphyxia. The results of an experiment of this type in which insulin was given at the height of epinephrin hyperglycæmia is shown in the following table:—

Sugar per 100 c.c. Blood.					Remarks.
Femoral Artery.	Femoral Vein.	Difference F.A.—F.V.	Portal Vein.	Difference F.A—P.V.	
Mg.	Mg.	Mg.	Mg.	Mg.	
232	224	8	216	16	After ether and operations.
232	219	13	228	4	10 minutes later.
264	244	20	271	+ 7	,, ,, after adrenalin.
318	282	26	298	20	30 ,, ,, ,,
292	286	6	291	1	60 ,, ,, ,,
309	288	21	293	16	12 ,, ,, insulin.
285	260	25	266	19	30 ,, ,, ,,
213	195	18	208	5	50 ,, ,, ,,
213	199	14	208	5	60 ,, ,, ,,
170	166	4	174	+ 4	90 ,, ,, ,,
168	157	11	140	28	120 ,, ,, ,,
—	—	—	—	—	B.P., 35 mm. Hg.
—	—	—	—	—	Glycogen, 0·36 per cent.

It can be seen that neither during the rise of the blood sugar, due to epinephrin, nor during its fall following insulin, can significant changes be observed in the various bloods. As a result of these and numerous other similar experiments, we feel very dubious whether observations of this type can be expected to yield satisfactory results on anæsthetised animals, even under the most carefully controlled conditions.

Cori, Cori, and Goltz have made similar observations on rabbits by a method which obviated the use of anæsthesia.

They used the following ingenious method for getting blood from the liver vessels. In a preliminary operation, the day before that of the actual experiment, an abdominal window was made and the falciform ligament of the liver cut. This allowed the viscus to fall away from the diaphragm when the animal was placed at an angle of 50° to the table. Blood was removed from one of the four hepatic veins by a syringe with

a special needle bent at its end, introduced through the abdominal window.

The average difference between the bloods of the hepatic vein and neck vein in eight rabbits was found to be 28 mgs. per 100 c.c. (minimum 24, maximum 33). In the five experiments in which insulin was injected, the above difference was decidedly less in two, but no different, or greater, in three. But closer scrutiny of the published results does not seem to justify the conclusions that insulin can sometimes diminish the sugar discharged from the liver. For example, in one of the experiments which shows a decrease (No. 2), only 1 unit of "iletin" had been injected one hour previously, whereas in the three other cases of this group, in which 10 units were used, there was either no change or evidence of an increased discharge. This leaves only one experiment in which the results indicate that insulin may have depressed the glycogenolytic process.

Since the foregoing comparison does not allow for retention of sugar by the tissues drained by the neck vein, the observations were repeated by using blood from the femoral artery and hepatic veins. At the same time, blood was also taken from the femoral veins, so as to show possible changes in the sugar-retaining powers of the muscles. The average percentage difference in sugar between bloods removed from the hepatic vein and femoral artery in nine normal animals was 23 mgs. (maximum 26, minimum 20), and in four out of five experiments a decided decrease was observed in one hour or more after the injection of insulin. The average percentage difference in sugar between bloods removed from the femoral artery and vein in twenty observations was 8 mgs. (maximum 13, minimum 3). In the five experiments just alluded to, this difference became significantly greater (showing increased retention of sugar) in three, remained practically unchanged in two, and became less in one, within a reasonable time (two hours) after insulin.

There are, besides, seven observations in which the blood sugar of the femoral artery and vein were compared; in three of these the normal difference was exceeded, and the authors' conclusions are "that several types of insulin action can be distinguished" . . . "both the liver and the muscle are influenced simultaneously" or "the liver alone seems to be responsible" or "the muscle alone is influenced." It is unfortunate that the comparisons in this careful piece of work were not made at

shorter intervals after the injection of insulin, for it is within the first half hour in starved rabbits, and not after an hour, that the greatest differences would be expected to show themselves.

Cori, Pucher, and Bowen (1923) have also compared the sugar concentrations in the arterial and venous blood of diabetic patients during insulin action, and have found, in six out of seven cases, that this caused a larger intake of sugar by the muscle.

Frank, Nothmann, and Wagner (1923) compared the percentages of sugar in bloods taken from the femoral vein and the heart of unanæsthetised rabbits. In normal animals the difference averaged only 4 mgs. per cent., but shortly after the injection of insulin into the femoral artery it rose as high as 50 mgs., to assume the normal difference after one hour.

The same workers also studied the effect on the sugar of arterial and venous blood of intra-arterial injection of insulin in normal and depancreatised dogs, and the results of the latter observations are particularly instructive. Whereas in normal animals there was slightly more sugar in the arterial blood, this relationship was the reverse in three out of five diabetic animals, i.e. the femoral vein blood contained more sugar than that of the artery. For about twenty minutes after injecting insulin this difference became greater (due to the arterial sugar falling more quickly), and then the venous blood sugar began to decline very rapidly, so that it came to be 20-50 mgs. per cent. lower than the arterial. The authors conclude that this shows that the sugar consumption in the muscles is depressed in diabetes.

In samples of blood taken simultaneously from the base of the finger-nail and a vein, the difference in sugar content is only small, in normal or diabetic individuals. After the ingestion of sugar, however, the difference becomes marked in the normal person, but not so in the diabetic. If insulin is given to the latter, the difference observed in normal persons becomes established (Lawrence). The finger blood is taken to represent arterial blood.

Faber (1923) and Wertheimer (1923) also found that the difference in the percentage of sugar in finger blood and vein blood, which occurs in normal individuals in favour of the former, is not present in diabetic patients, but becomes so after insulin. Taking these results as a whole, they confirm the

conclusion, arrived at from other data, that insulin accelerates the rate at which the muscles absorb sugar from the blood stream, but they do not satisfactorily demonstrate whether the liver assists in bringing about the hypoglycæmia by discharging less sugar.

The Effect of Insulin on the Rate of Disappearance of Sugar from the Circulating Fluid in Perfused Organs.—In contrast with the preceding, this method is one which is highly satisfactory for demonstrating whether or not sugar is being retained or produced by an organ, because the sugar either accumulates, or decreases, in the perfused fluid, and therefore causes readily measurable changes in percentage. Provided the experimental conditions are similar, the rate of sugar disappearance in the perfused heart of the rabbit or cat varies within narrow limits. In that of the rabbit, for example, the following results are recorded :—

Milligrams of Dextrose disappeared from 1 gm. heart per hour.	Investigators.
1. 1·5	Locke and Rosenheim (1907).
2. 0·5-1·0	Maclean and Smedley (1913).
3. 2·2	Mansfield (1914).
4. 0·7-1·6	Underhill and Prince (1914).

Under certain conditions the rate may be outside these limits. Camis (1908), for example, failing to detect any disappearance, whereas Gayda (1912) reported it to be as high as 7·1 mgs., but the perfusion conditions in their experiments cannot have been the standard ones.

Various investigators have studied the effects of pancreatic extracts on the rate of consumption of sugar by the isolated heart. Thus, Maclean and Smedley, using the heart of the diabetic dog or cat, found that the addition of pancreatic extract to the perfusion fluid increased the previously depressed sugar consumption, in some cases, up to normal, and, as is well known, Knowlton and Starling (1912) thought that the weakened sugar-consuming powers of the surviving heart of diabetic dogs could be greatly increased by pancreatic extract. In a later paper, however, Patterson and Starling (1913), showed that the sugar consumption of the diabetic dog's heart is the same as that of a normal dog's heart. The most important of the recent work is that of A. H. Clarke. Working with dogs, this investigator found that the perfused pancreas consumed no sugar, as judged by changes in the perfusion fluid, but that the amount consumed by the heart was very decidedly increased when the nutrient fluid was first of all perfused through the pancreas and then through the heart, or when the two organs were perfused in series. As a result of Clarke's investigations there can be little

doubt that the pancreas delivers, into the fluid perfused through it, something that accelerates sugar consumption by the heart of the same animal.

When insulin was finally obtained in concentrated form, its effect on the rate of sugar consumption by the perfused heart was investigated by Hepburn and Latchford. The heart of the rabbit was perfused according to the recirculation scheme devised by Locke, in which the fluid, after collecting in a small vessel placed under the heart, is raised through a narrow glass tube to the inflow reservoir by means of bubbles of oxygen. A total of 150 c.c. of fluid was used for each heart, and samples of 1 c.c. each were removed at intervals for determination of sugar by the Shaffer-Hartmann method. The pH of the fluid, the heart rate, and the volume flow were closely watched and great care was taken to keep the temperature constant in the various observations. Each experiment lasted about four hours, during which time it was found, in confirmation of Locke and Rosenheim, that there was no significant loss of sugar due to glycolysis or bacterial action in the perfusion fluid by itself. A precaution we consider of sufficient importance to mention is that only the purest distilled water [1] was used in making up the Locke's solution, since it was observed in this laboratory by the late T. G. Brodie that when solutions made with the ordinary distilled water were used for perfusion, neither the heart nor the intestine could survive for long. The ordinary distilled water may contain traces of substances produced in some way as a result of the chlorination process, by which the water of the lake cities is treated.

Twelve observations were made on hearts without insulin, with the results shown in graphic form in Fig. 29, in which the white columns give the milligrams of sugar consumed per gram heart per hour, and the other, variously shaded columns, the pH, the average rate of flow, and the glycogen content. For all observations without insulin, the average rate of sugar consumption is 0·87 mgs. per gram heart per hour, the maximum being 1·5 mgs. The average glycogen content—omitting one case in which it was very exceptionally high—was 0·207 per cent.

In the cases in which the influence of insulin was studied

[1] Water distilled from melted snow or from a deep spring was used.

(Fig. 29, II.) this was added to the perfusion fluid in various ways :—

(*a*) It was added to the perfusion fluid and the pH adjusted before the experiment. This method was used in the earlier experiments, but was not found to be entirely satisfactory.

(*b*) It was added to 10-20 c.c. perfusate removed about one hour

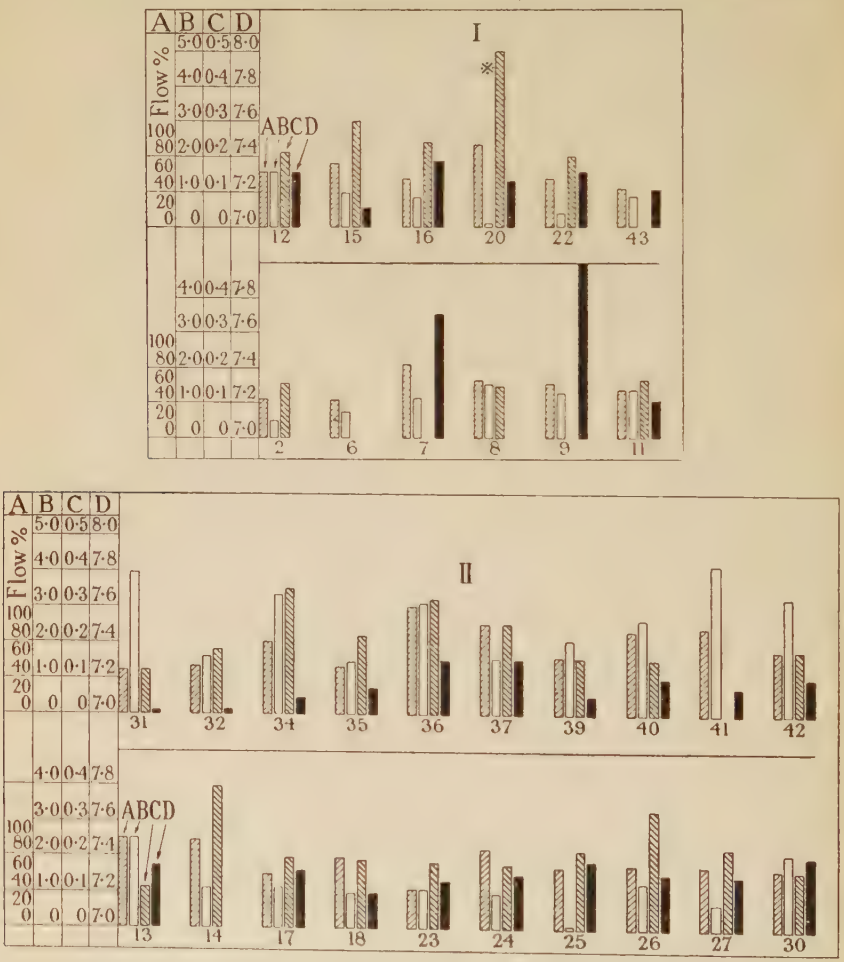

FIG. 29.—Chart showing rate of consumption of sugar by the heart. Fig. 1, when no insulin is added to the perfusion fluid. Fig. 2, when insulin is added. In each case A gives the average flow as a per cent. of the flow to start with; B, glucose used in milligrams per gram. of heart per hour; C, per cent. of glycogen in the heart; D, the pH of the perfusion fluid. The actual values of each of these are indicated at the left-hand margin. (Hepburn and Latchford.)

after the beginning of the experiment; the pH was then adjusted and the fluid replaced in the apparatus.

(*c*) Minute quantities, 0·1-0·25 c.c. insulin were added to the perfusate every fifteen minutes. This method was adopted in the later experiments, and was found to be the most satisfactory one, as it kept the pH at about 7·2, instead of increasing, as this otherwise tended to do.

(*d*) In several of the later experiments a dose of insulin was given subcutaneously twenty to sixty minutes before killing the animal and perfusing the heart.

The results of this series are shown graphically in Fig. 29 (II.). In the earlier experiments (13-27), as well as in experiments 32, 35, 36, and 37, the insulin was either of unknown strength, or was found, by testing it on rabbits, to be very weak. The sugar consumption of this group varied from 0·4·11 mgs., with an average of 1·9 mgs., and the glycogen content varied from 0·117-0·397 per cent., with an average of 0·215 per cent. In eight of the later experiments, in which the insulin was proved to be of high potency and free from protein, the consumption was as follows :—

Experiment.	Sugar Consumption per gram heart per hour.	Glycogen per cent.
	mg.	
30	2·16	0·160
31	3·90	0·120
34	3·30	0·350
36	3·06	0·311
39	2·06	0·153
40	2·58	0·155
41	4·11	lost.
42	3·29	0·179
	Average 3·06	0·204

Kymographic tracings were taken of the heart in several of the experiments, with the object of detecting any pharmacological action of insulin, but beyond a slight improvement in flow, no definite effect was observed. The average rate of flow and the pH were practically the same in the insulin, as in the control observations.

There is undoubted evidence that insulin greatly increases the rate at which sugar disappears from the perfusion fluid. In five cases this increase amounted to more than double the maximum observed in the series of twelve hearts perfused without

insulin, and in two cases it amounted to more than four times. It was not possible to account for the increased sugar disappearance by greater cardiac activity, for although this was not precisely measured, it was sufficiently observed (by counting the beats and measuring the outflow) to warrant this conclusion. As to the fate of the disappearing sugar—whether completely oxidised by the heart, or broken down by an anoxybiotic process, or polymerised into some polysaccharide, such as glycogen—cannot be said, although Hepburn and Latchford obtained some evidence that glycogen formation did not occur. Thus the average of the percentages of glycogen found in the normal hearts was 0·207, and that in the treated hearts 0·204, there being, however, considerable variability in the individual results.

Burn and Dale (1924) have repeated Hepburn and Latchford's experiments, with several modifications, introduced so as to make it possible to ascertain whether the additional glucose removed under insulin is used immediately as a source of energy or is stored in the heart in some form. In order to reduce the time required to bring about readily measurable changes in the concentration of sugar in the perfusion fluid, these workers limited the volume of the perfusion fluid to 50 c.c., this being rendered possible by the practical elimination of frothing by making the delivery tube from the oxygen-lift open on to a spiral of stout silver wire smeared with vaseline. By this modification it was possible, in some of the experiments, so to curtail the time necessary for perfusion that observations could be made on the same heart during a normal and an insulin period. The CO_2 produced by the heart was measured by passing the excess oxygen from the small collecting reservoir through a series of weighed soda lime absorption tubes. In five "normal" hearts perfused with Locke's solution the average glucose consumption, per gram of heart per hour, was 1·36 mgs., and the CO_2 production, 3·12 mgs. In five hearts in which one in 500,000 parts of insulin hydrochloride was added to the perfusion fluid, the corresponding values for glucose were 3·44 mgs., and for CO_2 4·66. Since the increase in CO_2 production is proportionately less than that of the disappearance of glucose, the loss of sugar cannot be entirely due to increased oxidation. By expressing the amount of CO_2 actually collected as a percentage of the amount which would be produced by complete combustion of

the glucose which disappeared, the interesting fact was brought to light that insulin caused a greater proportion of the CO_2 to be derived from the glucose. Thus, in the five normal hearts the percentage yield of CO_2 was above 100 in all cases, save one: whereas in the five insulin hearts it was well below 100 in all cases, save two. The " normal " hearts had evidently derived part of their energy needs from sources other than the glucose which disappeared from the perfusion fluid, in contrast to the insulin hearts, which derived all their needs from this source. Although with insulin the beats were more vigorous and rapid than without, the difference was not sufficient to account for the increased disappearance of glucose.

Confirmatory results were obtained when a mixture of the same animal's blood and Locke's solution was used in place of Locke's solution alone, the CO_2 percentage figures being very striking in this series.

In the following two experiments, normal and insulin periods were run on the same hearts :—

	Dextrose.	CO_2.	Per cent yield of CO_2.	Rate.
I. (*a*) No insulin.	4·23	5·34	86	162-200
(*b*) Insulin .	5·64	4·55	55	136-172
II. (*a*) No insulin	3·94	5·16	88	154-187
(*b*) Insulin .	3·03	2·45	55	90-164

It is clear that the increased disappearance cannot be attributed to increased beating, for in both cases this was not only slower, but also less powerful in the insulin period than in the control. The actual reduction in CO_2 during the insulin periods is significant, particularly in the first experiment. When experiments of a similar nature were done on the hearts of depancreatised cats, results similar to those of normal animals were obtained.

In summing up, Burn and Dale state " that some of the additional dextrose that disappears under insulin is not oxidised; the point that is not settled beyond question is whether any of it is." It was in order to answer this that experiments were made on eviscerated animals (p. 301).

Although the more rapid disappearance of sugar from the perfusion fluid could not be correlated with increased activity

of the heart in the foregoing experiments, Plattner (1924), using the Starling heart-lung preparation, has found that the addition of insulin does not increase the rate of disappearance of blood sugar so long as the heart rate is not increased. When this occurs the sugar consumption became greater in proportion to the increased heart rate. In one of the experiments in which 60 units of insulin were added to about 600 c.c. of blood mixture (1 unit in 10 c.c.), the rate of sugar consumption increased from 2·70 mgs. per gram heart per hour to 3·9 mgs., and that of the heart from 138-200 beats per minute. We are at a loss to explain this result.

We have attempted to measure the rate of the disappearance of sugar in perfused preparations of the skeletal muscles, but the great technical difficulties encountered in carrying out these experiments have made this impossible. For some purposes perfusion preparations of mammalian muscles may be of value, but they are useless for the determination of the rate at which substances are used up from the perfusion fluid. The considerable disorganisation of the arterio capillary tone, the entire paralysis of the muscles themselves, and the inevitable imperfections in the perfusion fluid, when compared with normal living blood, are probably responsible for these difficulties.

Location of the Action of Insulin by Studying its Effects after Elimination of Various Organs.—The observations of Mann and Magath (p. 302), in which it was shown that the blood sugar following insulin falls in dogs after removal of the liver at a rate which is comparable with that in normal animals, indicates that this viscus cannot be the chief locus of insulin action. The results do not, necessarily, prove that in the intact animal, the liver may not participate in the removal of sugar from the blood, for it is notorious how divergent the blood sugar curves following insulin may be in different animals, and it could only be after large numbers of observations had been made that any comparison would be justified. The experiments prove definitely however, that the liver does not play any essential rôle in the insulin effect. They rule out the possibility, suggested by Winter and Smith, that the liver acts with insulin to prepare the sugar molecule for utilisation.

Burn and Dale have attacked the problem by use of the decapitate cat, in which, as had previously been shown in this

laboratory by Olmsted and Logan, insulin develops its usual hypoglycæmic effects. By continuous injection of glucose solution, Burn and Dale augmented the rate of the disappearance of sugar several times, and found that complete removal of the abdominal viscera, but leaving the liver *in situ*, did not affect the results. In one preparation the skin was also removed without causing any difference. Since the addition of glucose in these experiments greatly increased the rate at which sugar disappeared (see p. 258), conclusive proof is furnished against the hypothesis, made without any evident justification by Laufberger, that insulin acts merely by stopping gluconeogenesis from fat, the sugar already present disappearing at the normal rate.

In light of these experiments there is little doubt that the chief locus of insulin action is the cardiac and skeletal muscles, and the question which arises is, what becomes of the sugar that disappears. In seeking the answer to this question, which is really the key of the whole insulin problem, one naturally proceeds from the known to the unknown, and in the first place proceeds to ascertain whether changes occur either in the amount of glycogen deposited in the body, or in the rate of combustion of carbohydrate, which are of sufficient magnitude to account for the sugar which insulin causes to disappear. In the event that this is found not to be the case, changes in other possible types of process that may be responsible must then be sought for. These questions have already been considered (Chaps. XII. and XVI.), and in the following pages we will review some of the other possibilities which have been investigated.

The Effect of Insulin on the Tissue Sugar.—Our knowledge of the concentration of sugar in the solid tissues is very limited, because of the numerous difficulties encountered in determining it. These difficulties are partly technical, and concerned with the extraction of the sugar and the removal of protein and non-carbohydrate reducing substances (rest reduction) from the extracts, and partly physiological, due to the presence in the tissues of hydrolytic, and other enzymes which become very active immediately the circulation ceases, and so cause changes in the concentration of free sugar. As a matter of fact, it has been found, by several workers, that it is impossible, by analysis of the entire animal, to account for more than a fraction of the amount of glucose injected into it shortly before death. The

injected sugar, as Claude Bernard first showed, only temporarily raises the blood sugar, and although, after the injection of strong solutions, changes affecting the water content and excretion of sugar by the urine may account for some of the decline in concentration which soon sets in, there can be no doubt that most of the sugar goes into the tissues. What happens to it there? Does it remain for any length of time as free sugar, or is it polymerised into some storage form, such as glycogen, or built up into some complex, such as hexose phosphoric acid, or is the change a more complete one, so that non-carbohydrate substances related to the fatty acids are the result? At present there is no satisfactory answer to these questions. The increased migration of sugar into the tissues, as Graham Lusk has shown, stimulates greater combustion of carbohydrate, but this can account for only a small fraction of the amount that disappears.

Extraction of the tissues with boiling water has usually been the method employed in investigating these problems, proteins being removed from the extracts, either with alcohol or phosphotungstic acid, which latter also removes substances, such as creatinin, which interfere with the reduction reaction. Von Brasol (1884) recovered less than three-fourths of sugar injected shortly before death. In 1913 Bang, by similar methods, recovered about three-fourths when the injection of sugar was made rapidly, but only one-half when it was made slowly, and none of that which disappeared could be recovered by acid hydrolysis of the extracts. In 1916 Kleiner modified the procedure by using anterior animals (the aorta and vena cava tied off at the level of the diaphragm), and by removing a fore leg prior to the injection of sugar. The reducing substances were then compared in extracts of the muscles of this leg and of the remaining one following the injection of sugar, allowance being made for the sugar present in the blood. Over 80 per cent. of the quantity injected could be accounted for in two, out of three experiments. Even when sugar was injected into recently killed animals, only 55 per cent. could be accounted for after fifteen minutes. A year later, Palmer, after a thorough trial of available methods for the extraction of sugar from the tissues and the removal of protein, etc., was able to recover from them rather more of intravenously injected sugar than his predecessors. Thus, in half an hour after the injection of 24 gms., 63 per cent. was accounted for, and in one and a half hours after the injection of 62 gms., 92 per cent. Other results were that after 76 gms, 70 per cent. was recovered, after 52 gms. 61 per cent., and after 40 gms. 55 per cent.

Interest in this problem has been greatly increased by the impossibility of explaining the rapid and extensive disappearance

of sugar from the blood following the injection of insulin. Inability to account for it by increased formation of glycogen or greater combustion have focussed attention on the concentration of free and combined sugar in the tissues. Among the first to attack this problem were Cori, Cori and Pucher. They first of all compared the free sugar of the liver, measured by Palmer's method, with the blood sugar in rabbits and guinea pigs, which were either previously starved or fed with glucose. With an increase in blood sugar, there was always a decided rise in the free sugar of the liver and, simultaneously, an increase in glycogen. After insulin, on the other hand, the free sugar fell parallel with that in the blood, but glycogen was still formed. Later Cori and Cori found that with an average fall in blood sugar of 54 per cent., the free liver sugar was reduced by 40 per cent. The free sugar of the liver, in seven mice that were killed by stunning and immediately frozen, averaged 0·300 per cent. (maximum 0·393, minimum 0·167); and in seven others, previously injected with insulin (forty-ninety minutes), the average was 0·167 (maximum 0·242, minimum 0·116). A certain allowance must be made for the blood remaining in the liver, but when this is done there is still evidence that insulin reduces the free sugar of the liver.

In only three, out of eight experiments, in which the muscles were examined could any decided reduction in free sugar be detected, in four there was practically no change, and in one there was a decided increase. Basing their conclusions on the average of these somewhat variable results, the authors state that insulin does not lower the free sugar of the muscles. On the other hand, this was found to be consistently and decidedly reduced in the kidney.

The problem has also been investigated in my laboratory in association with Eadie, Noble, and Orr, particular attention being given to a comparison of the free sugar of the muscles and liver of starved rabbits with that of rabbits injected with insulin, or with this *plus* sugar.

To prevent post-mortem glycogenolysis, or other changes that might affect the concentration of sugar, the tissues were instantly frozen by liquid air after their rapid excision, immediately following death by stunning. The brittle frozen mass was pulverised in an iron mortar, and after rapid weighing, 70 per cent. alcoholic extracts were

prepared, either at boiling, or freezing temperature. When the latter method was used, the extracts were rapidly centrifuged, rather than filtered, and great care was taken to avoid any rise in temperature, at least until after the extracts had been decanted. The decanted extracts were evaporated in flat dishes in a current of dry air.

It was observed, both in muscle and liver, that cold alcohol extracted more reducing substance than hot, which may indicate that heat destroys some of this material. Portions of the extracts were hydrolysed, with varying effects on the reducing power. In those made from muscle by hot alcohol, this always increased, but it might decrease after hydrolysis in cold extracts. The effect of hydrolysis on the liver extracts, by both methods, were very irregular, causing sometimes an increase and sometimes a decrease in reducing power, the latter especially in cold extracts. These results afford further support for the presence of highly labile substances in the extracts. After insulin there was less reducing substance in both liver and muscle, not only before, but also after hydrolysis. Following insulin and sugar, cold extracts of muscle contained more free and combined reducing substance than the controls, but considering the large quantities of the latter that were injected, the differences were very small, indicating that after its absorption by the tissues sugar is very quickly converted into some non-carbohydrate substance. There was some evidence in similar extracts of liver that the polymerised carbohydrate was increased after sugar and insulin, but it is possible that this may have been due to glycogen not thrown down by centrifuging. Although the results indicate that insulin causes the free and combined sugar that is soluble in hot alcohol to become reduced in both liver and muscle, they are not satisfactory, and the whole problem awaits further investigation. So far it has been impossible, by methods of extraction, to account for the very large quantities of sugar which disappear into the tissues when insulin is given. It looks as if some hitherto unidentified substance were formed out of the sugar, a substance possessing no reducing properties, either before or after hydrolysis, and which is readily destroyed at ordinary temperatures.

The Effect of Insulin on Lactic Acid, Acetaldehyde, and Related Substances.—Reference is made elsewhere to the impossibility of accounting for any of the sugar by the formation of

such a compound as lactacidogen (p. 334). Not unnaturally, considerable attention has also been given to the behaviour of lactic acid following the injection of insulin. At an early stage in the investigations of this substance, we measured the percentage of lactic acid in the blood of etherised dogs before and after causing the blood sugar to fall by means of insulin, without finding any increase beyond that which we had become accustomed to expect, in numerous observations on the behaviour of this substance in animals kept for some time under ether (Hepburn and Latchford, etc.). Briggs, Koechig, Doisy, and Weber later published results from which they concluded that lactic acid accumulated in the blood as the free blood sugar decreased. Isaac and Adler and Tolstoi and others also found that lactic acid sometimes increased in the blood of normal and diabetic men, following insulin, but not in amounts sufficient to account for the disappearing sugar. Best and Scott submitted the problem to a thorough investigation on laboratory animals with consistently negative results, and, indeed, it is evident on *a priori* grounds that accumulation of this substance in the blood could not be a significant factor in accounting for the very large amounts of sugar which are known to disappear, as for example, when sugar and insulin are injected together. Neither does lactic acid accumulate in the muscles. This was first of all shown in 1923 by H. W. Dudley (private communication), who determined the lactic acid in the muscles of rabbits both immediately after death and after standing some time. The yields from insulin-treated animals were very much less than those from normal ones. Kuhn and Baur obtained exactly similar results, the lactic acid of the muscles of rabbits killed by overdosage with insulin being about one-half that of normal (starved) animals, and only one-third when they were killed on the appearance of hypoglycæmic symptoms. The percentage did not increase on allowing the muscles to stand. These results are especially significant when taken along with the following facts: (1) That *rigor mortis* sets in with great rapidity following insulin convulsions and coma; (2) that watery extracts of these muscles, in previously well-fed animals, have an alkaline rather than an acid reaction (Baur, Kuhn, and Wacker, and (3) that the muscles of animals dying from hypoglycæmic symptoms contain only traces of glycogen. The amount of lactic acid in muscle does apparently

undergo a change following insulin, but it is in the opposite direction to the expected one.

We cannot leave this subject without reference to the work of Isaac and Adler, who are reported by Grevenstuk and Laqueur to have found that di-oxy acetone (CH_2OH CO CH_2OH), when given intraperitoneally to mice and rats, causes glycogen (glucose) to be formed, whereas when given to normal man (by mouth) it is converted into lactic acid, which appears in the blood and urine. Insulin increases this process. Given by mouth to severely diabetic men, on the other hand, di-oxy acetone is converted into sugar, until after treatment by insulin, when it forms lactic acid.

Another possible intermediary in sugar metabolism that is said to be affected by insulin is acetaldehyde. Neuberg, Gottschalk and Strauss, using the sulphite fixation method of Neuberg, found an increase, amounting to between 100 and 400 per cent. in liver pulp following the addition of insulin. A continuation of these investigations by Gottschalk (cf. Grevenstuk and Laqueur) revealed the fact that the yield of acetaldehyde, in mixtures of liver pulp and insulin, was greatly increased by also adding glycogen, but only moderately so by glucose or fructose, hexose-phosphates, dioxyacetone, and d.l. glycericaldehyde having intermediate effects. It is concluded that insulin stimulates the production of acetaldehyde, in contrast to epinephrin, which in certain concentrations has the opposite effect. Because of the probability that many of the intermediary products of carbohydrate metabolism are of a highly labile nature, and do not accumulate in sufficiently large quantities in the tissues to be detected by the usual chemical means, the principle used by Neuberg and Gottschalk for the detection of acetaldehyde is one which it is hoped may be found applicable to other intermediary substances. These results have been confirmed in my laboratory.

Insulin and Tissue Reductase.—A suggestive method for investigating the mechanism of the action of insulin is that introduced by Ahlgren. The reduction of methylene blue by tissues is believed to depend on enzymes called hydrogen transportases, which transport labile hydrogen from oxidisable metabolites (hydrogen donators) to the pigment, which therefore acts as a hydrogen acceptor. The time it takes for the methylene blue to be decolorised is considered to be a measure of the speed and

extent of the reduction processes by the tissues (Weinland, Thunberg). When the concentration of oxidisable metabolites is low, as in frequently washed muscle, decolorisation becomes much prolonged, but it can be accelerated again by the addition of various organic substances, including those believed to be intermediary in the catabolism of glucose (e.g. lactic acid), although this sugar itself has no effect. The tissues of normal animals, when only moderately washed, can convert glucose into a hydrogen donator, whereas those of diabetic (depancreatised) animals cannot do so, and the question arises as to whether this difference depends on the presence of insulin. Ahlgren has answered it in the affirmative by finding that the addition of glucose and insulin to tissue containing a sub-optional concentration of donator, or to diabetic tissue, results in an acceleration of bleaching time. Markowitz has repeated Ahlgren's experiments with the following results :—

The heart muscle of a rabbit was cut up with a sharp scissors in isotonic saline solution, and this was decanted and the chopped muscle washed several times and then pressed out, so as to lower the donator concentration and remove most of the insulin presumably present in it. A rapidly weighed portion (0·2 gms.) of the muscle was added to 0·2 c.c. of a buffer solution containing 1-1000 methylene blue, contained in a suitable vacuum tube which was evacuated. This was then placed, along with control tubes, as indicated below, in a water bath, and the time of decolorisation noted :—

Tube.	1	2	3	4
1 per cent. glucose .	0·2 c.c.	0·2 c.c.	—	—
m/10 lactate . .	—	—	—	0·2 c.c.
Insulin . . .	—	0·2 c.c.	—	—
Water . . .	0·4 c.c.	0·2 c.c.	0·6 c.c.	0·4 c.c.
Bleaching time in minutes . .	54	23	52	31

The water alone and the glucose took much longer to decolorise than glucose *plus* insulin, or than lactate, a known hydrogen donator. This confirms Ahlgren's results, and in numerous other experiments of a similar type with washed muscle of normal animals, this has in general been the rule, although we have been unable to satisfy ourselves that pancreatectomy, in frogs, makes any particular difference. At a certain concentration the effect of insulin is optimal. Thus in

one series of experiments, in which varying quantities were added to a solution containing glucose, the bleaching time increased up to a certain concentration, and then declined when more was present. Ahlgren also observed this, and it explains why negative results are sometimes obtained by the method.

Our interest in this work was aroused, partly, because of the possibility that it might serve as the basis for a test tube method for the assay of insulin, but we have met with no success in this direction. In the progress of this work our attention was called to the possibility that the reductase reaction of yeast might be used for a similar purpose, and as a matter of fact we did find that the decolorisation of a buffered suspension of yeast containing glucose was inhibited by the addition of commercial insulin in proportion to the amount added. Repetition of this experiment with the same insulin after it had been purified by Dudley's process (p. 71) showed, however, that the results had been due to impurities. It may be mentioned here that similar purification did not affect the results obtained on muscle by Ahlgren's method.

We do not consider that at the present stage any conclusions with regard to the physiological significance of Ahlgren's results are justifiable. They certainly cannot be considered as evidence that the hyperglycæmia in diabetes is caused by loss of power of the tissues to utilise glucose. Other interesting experiments, in which the influence of other hormones, such as epinephrin, pituitrin, and of various alkaloids, was investigated, have yielded results which may or may not be significant, but we cannot discuss these in detail here.

Nitzescu and Cosma (cf. Gravenstuk and Laqueur) have also repeated portions of Ahlgren's experiments, and among other things have found that insulin also accelerates the rate of decolorisation in the presence of oxybutyric acid, etc., a result also obtained by Rosling with watery extracts of pancreas.

Grevenstuk and Laqueur also refer in this connection to the researches of Heymans and Matton, in which the possible influence of insulin was studied on the Lipschitz reaction, which depends on reduction of M-dinitrobenzol by the tissues. Entirely negative results were obtained both by these workers and by Cloedt and van Canneyt. Neither could either of these groups of workers

confirm the findings of Buchner and Grafe that insulin can accelerate the production of CO_2 by isolated tissues.

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CHAPTER XIX.

THE EFFECT OF INSULIN AND OTHER HORMONES ON THE PHOSPHATES OF THE BLOOD, URINE, AND MUSCLES.

Marked changes in carbohydrate metabolism seem to be invariably associated with changes in the metabolism of phosphoric acid. This association must be more than a mere coincidence, and may indicate that phosphoric acid plays a rôle in the intermediary metabolism of carbohydrates, a possibility which becomes of greater interest when it is recalled that a hexose phosphate is formed during the fermentation of glucose, and that a compound of a similar nature is believed by Embden and others to be concerned in the phenomenon of muscular contraction. Fiske (1920, 1921) was the first to observe that a temporary diminution, followed by a compensatory increase, occurs in the output of phosphate by the urine during the ingestion of sugar. These changes are paralleled by similar ones in the blood. In cases also where the blood sugar has become raised, as in diabetes, or in alimentary hyperglycæmia, the intravenous injection of solutions of phosphates causes the blood sugar to become reduced in amount. These evidences of the relationship between phosphate and sugar metabolism have been greatly strengthened by the observation, first recorded by Wigglesworth, Woodrow, Winter, and Smith (1923), that insulin causes the inorganic phosphates of the blood to become decreased. This was confirmed by Blatherwick, Bell, and Hill (1923), by Perlzweig, Lathan, and Keefer (1923), and by Harrop and Benedict (1923, 1924) for the blood, as well as the urine of both normal and diabetic individuals.

The Urine.—In light of these observations it seemed important to determine the exact time relationships between the changes in the excretion of sugar and phosphorus, and those of the other urinary constituents following the administration of insulin

and the ingestion of sugar in normal and diabetic dogs. This work was undertaken by S. S. Sokhey and Frank N. Allan (1924).

After a preliminary period of three days' starvation, dogs were catheterised at intervals of about three hours during the day, the night urine being collected as a whole, and 50 c.c. of water were given by stomach tube at the beginning of each of the day periods and 150 c.c. at night. The observations started each morning at 10 o'clock, and the results of typical ones are shown in the chart of Fig. 29, in which the vertical lines correspond to three hour periods and the horizontal lines to the amounts of the various substances excreted per hour during each of the periods. The excretion of phosphoric acid is given in the heavy continuous line, nitrogen in the heavy dotted line, total acidity in the light dotted line, and ammonia in the dot and dash line. The figures standing above each of these lines represent the total excretion of the substance referred to during twenty-four hours, and the figures on the ordinates give the hourly averages.

On normal days, such as the 4th, 7th, and 10th of March (Fig. 30), it will be observed that the hourly excretion of phosphoric acid rose from a low point in the early forenoon to attain a maximum during the late hours of the evening. The nitrogen, on the other hand, was somewhat higher during the day than during the night, the highest value being usually reached in the early afternoon. The output of phosphorus for the night was considerably higher than that for the day, a fact previously observed by Fiske (loc. cit.) and by Campbell and Webster (1921). The ammonia and acidity varied in the same direction as the phosphorus.

When insulin was given during the forenoon (indicated by the arrows), as on the 5th, and again on the 8th, of March, the phosphate, instead of rising, fell promptly, so that it had almost entirely disappeared in from two to six hours after the injection. Then a remarkable increase occurred, so that in from nine to twelve hours after the injection it had risen to about 35 mgs., or more than three times the average normal hourly excretion. After this it again fell rapidly, so that by next morning it was about the usual level. During the twenty-four hours, in many of the experiments, nearly 50 per cent. more than the average excretion occurred.

In an observation on another dog, not recorded in the chart, it was found that the period of diminished excretion of phosphate could be prolonged by repeating the dose of insulin, although the increase

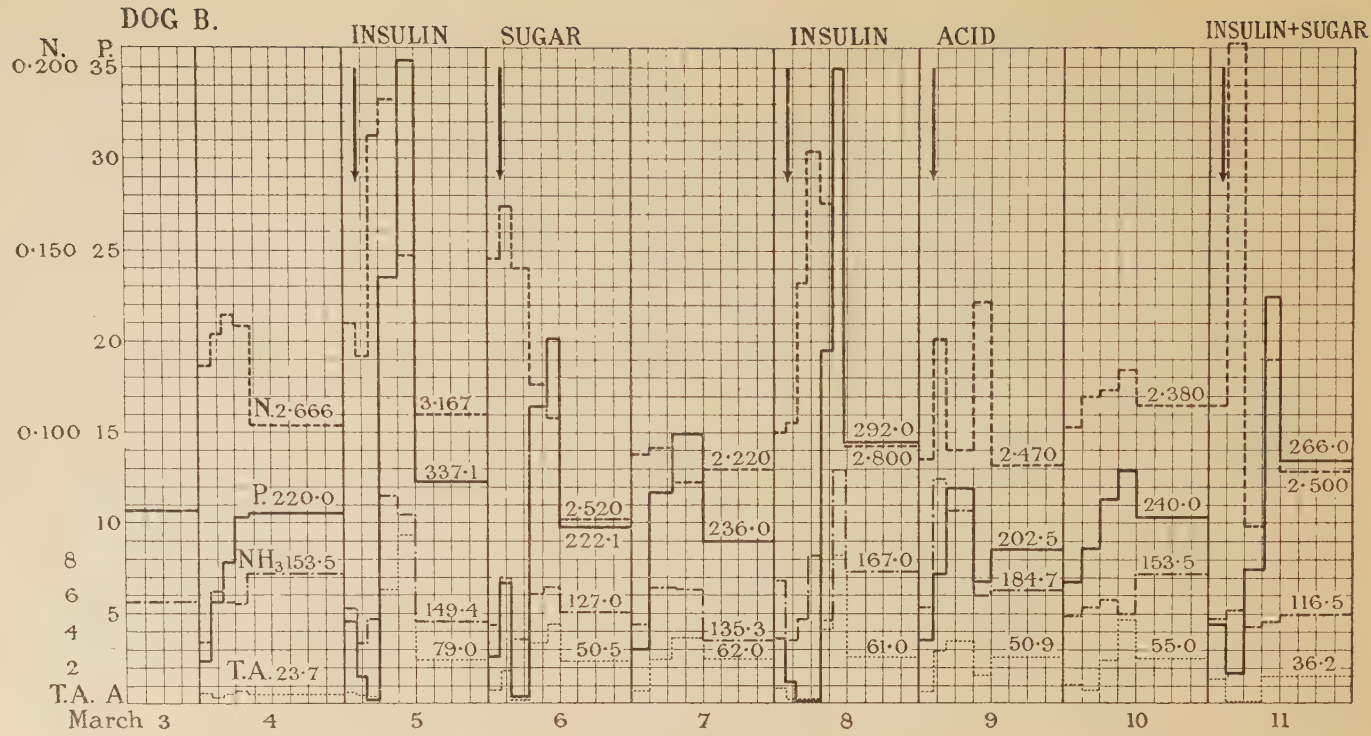

FIG. 30.—Chart showing effects of sugar and insulin on the excretion of phosphorus, nitrogen, etc., in normal dog (see context).
(Allan and Sokhey.)

ultimately occurred, even although it was evident that the effect of the insulin had not passed off, as demonstrated by the persistence of severe hypoglycæmic symptoms. In one observation of this type a compensatory rise in phosphates over-balanced the diminished excretion, so that in twenty-four hours the total was greater than the normal. In another case similarly treated, however, the total output for the day was diminished instead of being increased; and this was not compensated for by an increase in the phosphate excretion on the day following the injection.

Accompanying these changes in the excretion of phosphorus, it will be observed that the excretion of nitrogen increased during the period when the phosphate had almost disappeared from the urine, this increase continuing as the compensatory rise in phosphate occurred, after which the excretion fell to about the normal level, the daily output being, however, increased about 20 per cent. over the normal. In one of the observations, in which three injections of insulin were given, the daily output was approximately doubled. The excretion of ammonia and the titratable acidity of the urine approximately paralleled the excretion of phosphorus, except that, when the increase in nitrogen was very marked, the ammonia tended to fall relatively less than the acidity. Finally, it is important to note that the volume of urine was usually diminished when the phosphate excretion was low, increasing markedly again after six hours. In some cases this secondary diuresis occurred while hypoglycæmic symptoms were still urgent. It can, therefore, be stated that while the blood sugar is falling, as the result of insulin, the excretion of phosphate by the urine practically ceases, and that of nitrogen becomes greatly increased. This increase of nitrogen may indicate that sufficient preformed carbohydrate was not present in the body for insulin to act upon, so that protein became torn down to furnish more sugar. Possible disturbance of the acid base equilibrium of the body was not responsible for these changes, since, in the first place, this is very slight, if it occurs at all, after insulin (p. 254); and in the second, Fiske and Sokhey have shown that no changes in the phosphate excretion occur when acid is given to starving cats.

These results make it of interest to study, in the same manner, the effect of sugar. Such a result is shown in the chart for 6th March.

The dog was given 50 gms. glucose by stomach tube at the time indicated by the arrow. For two and a half hours after the ingestion the excretion of phosphate continued its usual forenoon rise, but then fell, so that it almost entirely disappeared, rising again in five hours, although not nearly to the same extent as after insulin, the total excretion for the twenty-four hours being not greater than the average normal output. It may, therefore, be stated that sugar has a delayed effect on the excretion of phosphates, similar to, though less marked, than that following a large dose of insulin. Perhaps the most interesting difference between the effects of sugar and insulin is with regard to nitrogen; instead of being increased by giving sugar, it gradually diminished, becoming very low during the night and remaining well below its normal level on the succeeding day.

These results may be interpreted as indicating that when insulin is given alone it not only uses up all the free sugar in the body, but also causes new sugar to be formed out of protein, a process which is prevented when sugar is given along with the insulin. This protein-sparing action of sugar probably explains the result previously obtained by Allan and Macleod (1923), that the daily excretion of nitrogen is diminished when moderate doses of insulin are given to well-fed animals.

When insulin and sugar were given together there was a temporary increase in nitrogen, although the amount excreted in twenty-four hours remained normal; the effect on phosphate excretion was in the same direction as, but less marked than, after insulin alone. The increased excretion of nitrogen following insulin alone was not found to be accompanied by a corresponding increase in creatinine, the only change observed to occur in the excretion of this substance being that in one case in which a second injection of insulin was given, so as to bring the animal into a state of collapse, it fell from about 6 mgs. per hour to 4·3 mgs. per hour. The failure of the creatinine to rise at the same time as the nitrogen would seem to indicate that the nitrogen cannot be derived from endogenous protein.

These significant results with insulin and with sugar indicated the advisability of studying, by similar methods, the effects of various other conditions known to upset the metabolism of the carbohydrates. Depancreatised dogs kept alive by insulin (see p. 77) were observed by Sokhey and Allan (Fig. 31). Food and insulin were withdrawn, and on the following day the excretion of phosphorus for the twenty-four hours was found to be more than double that of a normal animal, although it showed similar hourly variations. The nitrogen excretion was

also very high. When insulin was given (6th March) the phosphates fell immediately, reaching almost to zero in from five to seven hours, after which there was no compensatory increase, but only a moderate return, so that the excretion for the day was reduced to a value approximating that for normal animals.

When it was found that ingestion of sugar caused changes in the excretion of phosphates similar to those following insulin,

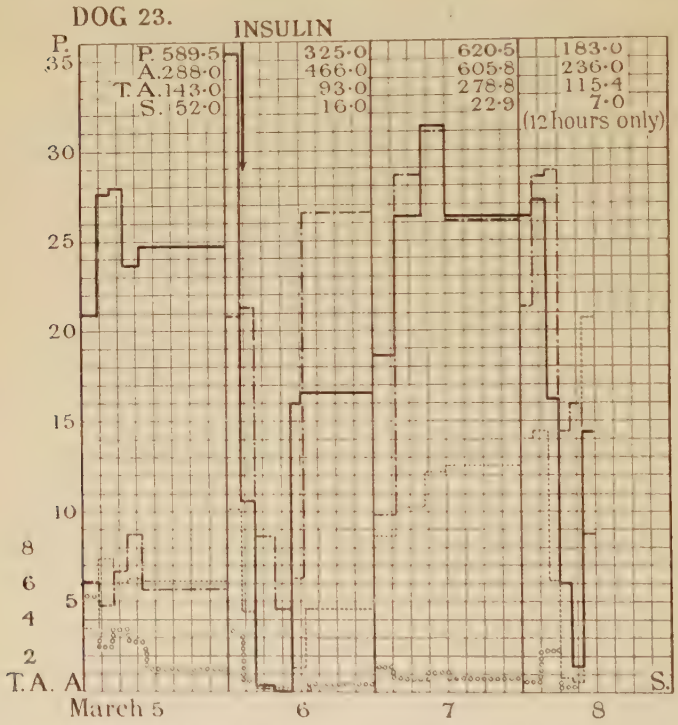

FIG. 31.—Chart showing effect of insulin on the excretion of phosphorus, nitrogen, etc., in a depancreatised dog. (Allan and Sokhey.)

though delayed, it was supposed that the sugar must act by stimulating the internal secretion of insulin from the pancreas. If this be the correct explanation, the ingestion of sugar should not cause the same changes in the excretion of phosphorus in depancreatised dogs as occur in normal animals. Markowitz has put the question to the test of experiment by using fasting depancreatised dogs several days after the withdrawal of insulin.

In one of the observations insulin was withdrawn on 6th July, and the hourly phosphoric acid excretion on 8th July for each of the usual three-hourly periods was 13·9, 14·6, 14·3, 16·7, and 14·3; on 9th July these figures were: 17·1, 16·2, 17·1, 17·2, 12·2. On 10th July about 20 gms. glucose were given, and the hourly quantities of phosphoric acid were: 14·3, 18·1, 20·8, 13·4, and 13·6. This observation was repeated on three other depancreatised animals with corresponding results, namely, that sugar instead of causing the excretion of phosphorus practically to disappear was followed by a slight increase. In

FIG. 32—Chart showing effect of epinephrin on the excretion of phosphorus, nitrogen, etc., in a normal dog. (Allan and Sokhey.)

one animal only did a slight decrease occur. Bollinger and Hartman had previously obtained similar results.

The clear-cut character of these results shows that the behaviour of the excretion of phosphorus in normal animals, following the administration of glucose, depends on stimulation of the internal secretion of insulin by the sugar.

Epinephrin also causes changes in the excretion of phosphorus and nitrogen similar to those of insulin (Fig. 32), except

that the secondary rise is not so marked and the increased excretion of nitrogen persists into the day following the administration.

When insulin and epinephrin are given together, the same changes in the excretion of phosphate and nitrogen by the urine occur as when either is given separately, as is shown in the experiment of 22nd May, although they neutralise each other in so far as blood sugar is concerned. Since insulin and epinephrin have opposite effects on the blood sugar, but similar effects on the excretion of phosphates, it is clear that they cannot be strictly antagonistic with regard to carbohydrate metabolism. It is possible that the same product of carbohydrate metabolism is produced by epinephrin, by insulin, and by excess of sugar ingestion for the formation of which phosphate is necessary. In passing, it may be remarked that the increase in nitrogen observed in these experiments on fasting animals, not only on the day on which epinephrin was given, but also on the succeeding day, definitely indicates an increase in protein metabolism, as had previously been observed by Eppinger, Falta, and Rudinger (1908), by Noel Paton (1903), and by others, although denied by Allen (" Glycosuria and Diabetes ").

The effect, on a fasting animal, of the daily injection of phloridzin is very similar to that of pancreatectomy.

Sokhey and Allan found that the phosphates steadily rose, with the usual diurnal variations, so that in three days after the injections had been started the total excretion for the twenty-four hours had increased approximately three-fold (2 June). By this time the animal was, of course, intensely diabetic, and the nitrogen, which had begun to increase on the evening of the first day on which phloridzin was injected, rose to more than twice its normal by the fourth. When the phloridzin was discontinued, excretion of both phosphorus and nitrogen immediately fell, so that after two days the phosphorus reached a level much below the normal. The main interest of these observations is that they parallel those found in depancreatised dogs, and it is significant that a similar excess of excretion of phosphorus has been observed in human diabetics by Mandel and Lusk (1904), and by others.

These facts suggest the possibility that in diabetes, both in its experimental and clinical forms, a deficiency of phosphorus may come to be established in the body, and in this connection it is of interest to recall that the intravenous injection of phosphate solutions in diabetes may be followed by a decided reduction of hyperglycæmia and glycosuria (p. 325).

Blood.—The striking relationships demonstrated between the effects of various diabetic agencies and of insulin on the phosphate

excretion by the urine calls attention to the behaviour of the phosphates of the blood under similar conditions. As has already been pointed out, Winter and Smith and others have found that insulin reduces the phosphates in blood, and more recently observations have been made in this laboratory by Eadie, Macleod, and Noble (1925), and by Chaikoff and Markowitz, on the exact time relationships between these changes and those of blood sugar and acetone in normal and diabetic dogs. The relationships in normal dogs are shown in Fig. 33, and those

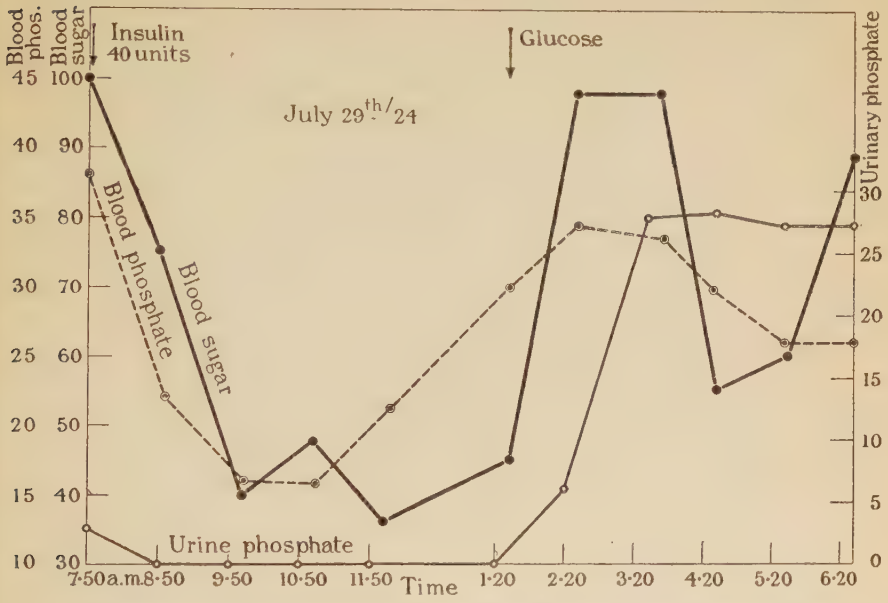

FIG. 33.—Curves showing the effect of insulin on the phosphorus (inorganic) and sugar of the blood in a normal dog. (Eadie, Noble, etc.)

on diabetic ones, in Fig. 12 on page 92. It will be seen, in both cases, that the inorganic phosphates of the blood decrease almost parallel with the blood sugar, but that the phosphate commences to rise decidedly before the sugar in the recovery process. The phosphate does not reappear in the urine until after it has been restored to a fairly high level in the blood, and it would appear that the threshold for phosphorus in the kidney is set at a definite level of 25-35 mgs. per 100 c.c. After the phosphorus begins to appear in the urine the usual augmented excretion occurs. It will be observed that glucose had to be

injected in the animal from which the results of the chart of Fig. 33 are taken, because of urgent hypoglycæmic symptoms. Although this resulted in a marked increase in blood sugar, it also caused a secondary fall in the phosphate in the blood, without any change in the phosphate of the urine. In rabbits, in from two to three hours after insulin, there was either a decrease or an increase in the blood phosphates, this variability being no doubt dependent on whether or not the secondary increase in phosphates had set in when the blood was withdrawn.

Muscle.—We might explain the foregoing results by assuming that phosphorus becomes united in some complex with the sugar entering the tissues, and that this complex later breaks down to release phosphoric acid. In other words, a compound analogous with that of hexose phosphate, described by Harden and Young as intermediary in the break down of sugar by yeast, might be formed. To test this possibility, we have examined to see whether the so-called *lactacidogen* described by Embden is affected by insulin. Embden, Laqueur, and their co-workers, believe that glucose is not utilised directly in muscle, but only after being changed into some form which is directly derivable from glycogen. They base this hypothesis upon the fact that when lactic acid is formed in muscle, so also is phosphoric acid in approximately equimolecular proportions. They regard the difference in the amounts of inorganic phosphates found before and after incubation of muscle excised immediately after death as a measure of the amount of this *lactacidogen* present in it. It seems possible, then, that insulin might cause *lactacidogen* to be formed, at least as an intermediary substance. To test this possibility we have compared, by Embden's methods, the *lactacidogen* of muscle of rabbits treated with insulin, or with insulin and glucose, with that of normal uninjected animals. In some of the observations Buchner extract of muscle was used, but in others, muscle frozen by liquid air and then broken up into minute fragments. As can be seen from the results shown in the following table, instead of there being an increase as the result of insulin, a slight decrease occurred. We feel confident that lactacidogen is not formed in muscle as the result of insulin, although Andova and Wagner, and Harrop and Benedict, have concluded that it is. The inorganic phosphates of the muscle, on the other hand, were increased in the animals injected with

insulin and glucose, but it is difficult to interpret the significance of this result.

TABLE XXII.

CENTIGRAMS PHOSPHORIC ACID PER 100 GMS. FROZEN MUSCLE.

Before Incubation.	After Incubation.	Lactacidogen Phosphorus.
Part I.—Normal Rabbits.		
34	55	22
31	44	13
29	46	18
42	58	16
38	65	26
42	62	19
Average 33	55	19
Part II.—Rabbits Injected with Insulin.		
41	50	9
34	47	13
28	47	10
31	46	15
47	62	14
32	53	19
30	56	25
35	45	9
Average 33	54	16
Part III.—Rabbits Injected with Insulin and Glucose.		
45	57	12
45	56	11
43	57	14
46	55	9
44	57	13
Average 44	56	12

Collazo and others (1924) have also failed to demonstrate that insulin and sugar causes an increase in lactacidogen. Observations on the amount of lactic acid in the muscles, both before and after standing, confirm these results. Indeed, instead of an increase, insulin causes the lactic acid to become markedly diminished. This was observed by H. W. Dudley, in June, 1923, and has since been completely confirmed by Kuhn and Baur (1924), and Collazo, Haendel, and Rubino (1924). It is obviously important in observations of this type that convulsions should be avoided, since, as Embden, Schmitz, and Meincke (1921) have shown, lactacidogen is diminished after strychnine convulsions. There is therefore at present no indication as to what becomes of the phosphorus that disappears

from the blood and urine following the injection of insulin, or during other disturbances in carbohydrate metabolism. Whether it plays an essential rôle in the process accountable for these changes is impossible to say.

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CHAPTER XX.

THE RELATIONSHIP OF DUCTLESS GLANDS OTHER THAN THE ISLES OF LANGERHANS TO CARBOHYDRATE METABOLISM.

Four ductless glands beside the isles of Langerhans, namely, the thyroid, the parathyroids, the pituitary, and the adrenal gland, have at various times been emphasised as having some important influence on carbohydrate metabolism. A great part of this work is of very doubtful dependability, but, nevertheless, it is certain that at least the thyroid and the adrenal glands have *some* such relationship, and probably also the pituitary. Fantastic hypotheses have been elaborated to explain the mechanism of this pluriglandular control, but it is only recently that indisputable facts have been recorded. It is with a few of these that we will concern ourselves at present.

The Adrenal Gland.—The main relationship of this gland and of its supposed internal secretion, epinephrin, have been discussed in various places of this volume. Thus, the relationship to pancreatic diabetes (p. 233), to the various forms of experimental hyperglycæmia (p. 241) and to insulin hypoglycæmia (p. 273), will be found in the chapters devoted to these subjects. The effects of epinephrin on the excretion of phosphorus has also been considered (p. 331). Recently J. H. Burn and H. P. Marks have observed that important changes in the hyperglycæmic effects of epinephrin result from thyroidectomy or thyroid feeding, and these we will consider in the next section.

The Thyroid Gland.—Although at various times it has been stated that the assimilation limit for sugar (p. 217) is significantly altered by thyroidectomy or thyroid feeding (for review, see Macleod, "Diabetes"), nothing very definite was recorded until 1913, when Cramer and Krause showed that the addition of thyroid gland to the diet of cats and rats caused glycogen to disappear from the liver, this being confirmed by Kuriyama.

Since, as we have seen, the recovery of the blood sugar following insulin hypoglycæmia is very largely dependent on mobilisation of sugar out of the glycogen stores of the liver, it becomes of interest to observe the influence of thyroidectomy and of thyroid feeding on the effects of insulin. This was first of all done by Bodansky, using sheep. The blood sugar in these animals is remarkably low, between 0·060 and 0·070 per cent., and it becomes lowered to about 0·040 within thirty-five minutes after the intravenous, but not the subcutaneous, injection of insulin (5-15 units), larger doses having scarcely any greater an effect than smaller ones, though the duration of their action is much longer (one and a half hours for 5 units, five hours for 15 units). Even the largest doses used were not observed to cause symptoms. Simultaneous intravenous injections of thyroxin and (5 units) of insulin had the effect of prolonging the hypoglycæmia without affecting its intensity, a similar result being obtained when the thyroxin preceded the insulin. On the other hand, when thyroxin was not injected until after the minimum blood sugar value had been passed, a decided degree of hyperglycæmia followed (blood sugar rose to 0·095 per cent.). Bodansky also found that insulin causes a more profound effect when given to thyroidectomised sheep; thus, after 5 units, the blood sugar continued to fall for a longer time, fell lower, and took longer to recover than in normal animals. Similar results have been obtained by Burn and Marks on rabbits, who also found that much of the irregularity in the response of these animals to insulin is eliminated by this operation. Ducheneau has also found that thyroidectomy increases the lethal action of insulin in rabbits.

Burn and Marks have added considerably to these observations by recent experiments, in which they have shown a much closer relationship to exist between the thyroid and the control of blood sugar than had ever been suspected. After thyroid extract has been added to the daily diet of a rabbit for a period of about a week, the animal becomes relatively insensitive to insulin, a dose ten times in excess of that which had caused convulsions before the thyroid feeding was started being now incapable of producing any symptoms. Evidently the glycogenolytic mechanism becomes more sensitive, so that mobilisation of sugar occurs at a lesser degree of hypoglycæmia

than that necessary to cause it in a normal animal. That this is the correct explanation for the result was evidenced by the fact that injection of epinephrin into a similarly thyroid-fed animal caused a greater degree of hyperglycæmia than the usual. When the thyroid feeding was continued for a longer period (ten to fourteen days or longer) a curious reversal of its influence on the effect both of insulin and epinephrin occurred, for, after this period, the rabbits became extremely sensitive to the former, and less so to the latter hormone. During both the early and the late phases of the thyroid influence there was an inverse relationship between the effects produced on blood sugar by insulin and epinephrin, and a similar relationship was also seen to exist in normal animals. Thus, as we have seen (p. 226), rabbits vary greatly in their extent to which epinephrin causes hyperglycæmia, just as they do in their response to insulin, and Burn and Marks have found that animals sensitive to the one hormone are insensitive to the other.

In the livers of animals killed during the first phase of thyroid feeding (lessened insulin effect), no significant change was found in the percentage of glycogen; during the second phase (increased insulin effect) the glycogen became less, and finally disappeared (in one animal after eighteen days). Evidently the development of increased sensitiveness to insulin depends on the disappearance of glycogen from the liver, and it is significant that under these conditions hypoglycæmia may occur spontaneously. Of still greater interest is the latest discovery of these workers, namely, that administration of glucose to a rabbit treated with thyroid for long enough so that it is hypersensitive to insulin, causes only a transitory rise in blood sugar, followed by a very rapid fall, which may become so marked that the animal develops severe hypoglycæmic symptoms. The administration of more sugar may only temporarily relieve these symptoms, by partial restoration of the blood sugar, but they soon recur, so that it is difficult to prevent death from hypoglycæmia. There can be little doubt that the first injection of sugar acts by stimulating the internal secretion of insulin from the pancreas, and that it is because of the effect of this, acting in an organism unprovided with the means of producing sugar from the liver, that the uncontrollable degree of hypoglycæmia is due. It is possible also that the prolonged feeding with

thyroid either uses up protein or fatty substances, which, in a normal animal, might provide the sugar after all the glycogen reserves had become exhausted. The gradual restoration of the blood sugar which occurs in completely deglycogenated animals after the injection of insulin must, as we have seen, depend on gluconeogenesis, and that such does not apparently occur under the conditions of Burn and Marks' experiment, may be due to an exhaustion of this process as a result of the thyroid feeding. It is possible also that another factor is involved, namely, that the internal secretion of insulin is rendered hypersensitive by some effect of the thyroid hormone on the cells of the isles of Langerhans.

The experiments afford strong support to the view held by so many that a close relationship exists among the ductless glands in the control of carbohydrate metabolism, and since we know that the thyroid also influences that of protein, the significance of these results can easily be seen.

The Pituitary Body and Pituitrin.—Burn has shown an interesting antagonism between insulin and pituitrin. Given in large doses intravenously, pituitrin, like insulin, may inhibit the hyperglycæmia due to epinephrin or to ether, although when given alone it may cause the blood sugar to increase. When pituitrin is given along with insulin it greatly diminishes, if, indeed, it does not entirely prevent, the fall in blood sugar. In other words, a dose of pituitrin which by itself would have no definite effect on blood sugar, may, nevertheless, antagonize entirely the hypoglycæmic action of insulin. Burn considers this to indicate a direct antagonism between the two hormones, which, as we have seen, cannot be considered to be the case between epinephrin and insulin.

It has frequently been observed that the blood sugar becomes much raised after decerebration (in cats), and it was considered possible that this might be related to a hypersecretion from the pituitary body. To test this possibility, Olmsted and Logan performed decerebration high up, that is, so that the cut passed in front of the corpora quadrigemina and did not wound the pituitary body. In such cases marked hyperglycæmia was invariably observed. In other animals, after the decerebration, the pituitary gland was removed by careful dissection, and it was found that although the blood sugar was high immediately

following the operation, it gradually fell to about the normal level. In a subsequent paper, Olmsted and Taylor refer to a suggestion by W. B. Cannon that the cause for the different effects on the blood sugar of high and low decerebrations may not be dependent on the presence or absence of the pituitary, but on whether or not the hypothalamus has been injured. In any case, it is interesting that insulin much more readily reduces the blood sugar when the pituitary is absent than when it is present. The following typical results will serve to illustrate :—

Cat No.	Blood Sugar Percentage at Hour Intervals after Decerebration.						
	1	2	3	4	5	6	
30	—	0·316	0·298	0·320	0·320	—	Pituitary intact.
5	0·230	0·270	0·300*	0·170	0·160	—	,, ,,
2	0·240*	0·240	0·235	0·200*	—	—	,, ,,
44	—	0·250	0·172	0·120	0·101	0·084	,, removed.
6	0·230	0·212*	0·124	0·080	0·058*	0·052	,, ,,
28	0·170*	0·136	0·084	0·072	—	—	,, ,,
29	*	—	0·048†	0·026 †	0·040†	—	,, ,,

* Insulin injected. † Convulsions occurred at this point.

With the pituitary intact it was not possible to lower the blood sugar sufficiently to cause convulsions, whereas this was readily done in the absence of the gland.

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CHAPTER XXI.

THE PHARMACOLOGICAL ASSAY OF INSULIN.

During the earlier stages of their use in medical practice there has usually been considerable confusion with regard to the dosage of the various glandular preparations that have a therapeutic value. Not only may the preparations of different manufacturers be assayed according to entirely different standards, but there may be considerable irregularity from time to time in the strength of the product of the same firm. With some glandular preparations no particular risk to health is incurred by these irregularities, but in the case of insulin, serious consequences would almost certainly follow were the strength of the preparations not known and uniform. The patient using feeble preparations would be living under a false sense of security, but a still graver risk would be incurred with unusually strong ones, because of the hypoglycæmic symptoms which have so frequently been referred to in this volume.[1]

In would be out of place in this volume to enter into the technical details of the bio-assay. We will content ourselves with a general review of the main principles involved, and with evidence indicating the degree of accuracy that can be expected.

The first question concerns the value of a unit of insulin. When it was found that characteristic symptoms usually super-

[1] At an early stage in the work on insulin, it was found that a suitable method of bio-assay could be worked out by using the rabbit as a test object and steps were accordingly taken by the University of Toronto to exercise what control it could over the manufacture of insulin of uniform potency for use in medical practice. It set up a testing laboratory for insulin produced in Canada and the United States, and it had the good fortune to have the co-operation of the Medical Research Council to exercise control in Great Britain. In various countries of Europe, also, testing laboratories were established. International uniformity in dosage has been further assured by conference among those testing authorities, through the Permanent Standards Committee of the Health Section of the League of Nations.

vene in fed rabbits when the blood sugar reaches the level of 0·045 per cent., 1 unit was defined as that amount which could produce this effect. This definition had soon to be modified, since it was found that the body weight and the amount of glycogen in the liver affected the results, and it was stipulated that animals of average weight (2 kg.) from which food had been withheld for eighteen to twenty-four hours should be used. The question now arose as to whether the behaviour of the blood sugar or the incidence of symptoms should form the basis of the assay, and for reasons which will be detailed further on, the former was chosen as the standard routine procedure. In using this method the question remained as to when, after injecting insulin, blood should be removed for analysis. The results of McCormick, Noble, and Macleod, referred to elsewhere (p. 270), showed that there was no fixed period during which the blood sugar was at its lowest in different rabbits, even when food had been withheld from them for some time. This made it evident that dependence could not be placed on the analysis of one sample of blood, and the expedient was adopted of taking the average of three results obtained in one and a half, three, and five hours after injecting the insulin. These intervals were chosen because in most rabbits the lowest level of blood sugar occurs after about the first of them, and recovery to the normal level at about the third. Another reason for using the average of several blood sugars was that it gives some consideration to the duration of the hypoglycæmic effect. At this stage it was stated that the unit, as originally defined, was too large for the treatment of certain cases of diabetes, and after some trial of different fractions it was decided to adopt one-third of the original unit as the standard.

At the first meeting of the Standardisation Committee of the Health Section of the League of Nations, a unit of insulin was provisionally defined as one-third of that amount which would lower the blood sugar of a normal rabbit of 2 kg. weight, and from which food had been withheld for twenty-four hours, to the convulsive level within three hours.

While this definition does not conform with the unit which is determined by taking the *average* lowering of blood sugar as outlined above, it has the great advantage of being one which can be accepted by those using either the incidence of convulsions

or one blood sugar estimation as the method for carrying out the assay. As a matter of fact, it is impossible that the exact value of a unit can be laid down in absolute terms, because of the variability with which different animals react towards insulin. For practical purposes the important thing is to have some standard with which the assay arrived at by any dependable method can be compared. A dry preparation of insulin hydrochloride has been prepared (by the Medical Research Council) for this purpose, and its assay has been found to be such that 1 mg. equals 8 units. This result, it may be mentioned, is based mainly on the degree of hypoglycæmia which the powder causes in rabbits, but it has also been obtained by comparing its convulsion-producing tendency (on mice) with that of another standard preparation.[1]

The international unit of insulin may therefore be defined as the quantity which produces an effect on carbohydrate metabolism equal to that of one-eighth of a milligram of the standard preparation of insulin hydrochloride. The introduction of this standard will not materially alter the assays of the various preparations of insulin in present use, for it has been carefully compared with these, so as to avoid such confusion, and it will fix a definite value to the unit for all time. It leaves open the exact method to be used in conducting the bio-assay, for it would be hazardous at the present time, when the physiological action of insulin is so little understood, to adopt any particular method for this purpose.

We will briefly outline the principles of the methods most commonly employed at present.

The Methods of Assay.—It may be explained that in all methods a preliminary assay is made to determine the approximate strength of the preparation.

1. *The Toronto Method.*—Each of three properly selected and prepared rabbits is injected subcutaneously with 1 c.c. of a solution, containing, as nearly as possible, 2·5 units of insulin (just short of a convulsive dose), three other rabbits with 0·8 c.c. of the same solution (equalling 2 units), and a third group of three with 0·6 c.c. (equalling 1·5 units). The concentrations of insulin being the same in all cases,

[1] This international standard will be kept under the auspices of the Health Committee of the League of Nations, and from time to time testing laboratories will have the opportunity of having their own standards compared with it.

it is presumed that the rates of absorption will be uniform, so that the differences between the doses will be manifested, not in the initial, but in the total effects which are produced over a period of time. This procedure was adopted because it was found impossible to detect any proportionality between the extent of the initial fall in blood sugar and the amount of insulin injected, unless very small doses were used (Macleod and Orr), and even then the proportionality was far from being a close one (see p. 272). With very small doses also there is much greater variability in the effects than with moderate ones, probably because a certain amount of insulin must be injected before there is any effect on the blood sugar. A certain threshold, in other words, must be reached, and the amount of insulin which has to be injected in order to do so will vary in different animals, according to the amount of insulin already present in the body. By injecting solutions of corresponding concentrations this threshold will always be overstepped, whereas with those that are of unequal concentration, insulin may be so slowly absorbed from the weaker ones that its concentration in the blood never reaches the threshold, although it may meanwhile influence the internal secretion of insulin from the pancreas, or affect other ductless glands having some control over the blood sugar level. Be the explanation what it may, there is no doubt that more constant results are obtained with the above method than when insulin solutions of varying concentrations are injected (Orr). Blood for sugar analysis is removed from each of the nine rabbits before the injections are made (normal) and in one and a half, three, and five hours after, the animals being meanwhile kept at a uniform temperature.

The unit value is then calculated from the following equation :—

$$\frac{a}{b} \times \frac{w}{c} \times 1{\cdot}5$$

in which—a = the difference between the average of the percentages of blood sugars after insulin, and the percentage immediately preceding the injection.

b = the difference between the percentage immediately preceding the injection and 0·045 (the convulsive level).

w = the weight of the animal in kg.

c = the volume, in c.c., of the actual preparation of insulin injected.

1·5 = a factor to allow for the fact that the unit is one-third of the amount which would lower the blood sugar percentage to 0·045 per cent. in a rabbit of 2 kg. weight ; therefore ($\frac{3}{2} = 1{\cdot}5$).

Although this equation is largely empirical, it has proved itself to be most useful in practice. The value a is the most significant one in it, but two fallacies are incurred in its determination. One of these has already been referred to, namely, that the average of the lowering,

and not the lowest blood sugar is taken. The other is that it assumes that the fall of blood sugar is directly proportional to the number of units of insulin, which obviously cannot be the case, for two units must have decidedly less effect than that of one unit multiplied by two. As the dose *c* increases, the extent to which the blood sugar is lowered (*a*) must become progressively less and less. When the relationship between *a* and *c* is plotted with *a* on the ordinates and *c* on the abscissa a curve is obtained which is steep at first but becomes more and more parallel with the abscissa as the dose is increased. It may be possible to make allowance for this source of error by determination of the exact equation of this curve, but for the present it is found to be most practical to endeavour to use such doses of insulin as will give effects falling towards the upper part of the steeper portion. It is interesting to note that a similar curve was obtained by F. N. Allan for the glucose equivalents of insulin.

Owing to the variable reaction of different rabbits to the same dose of insulin, the results obtained by the above method may not agree well with one another. If one result, in any of the groups, disagrees from the others by more than 25 per cent. between extremes, the corresponding dose of the same insulin is reinjected into three other rabbits, and this procedure is repeated until results agreeing within these limits are obtained. If the averages of the three groups do not agree within 25 per cent. the whole assay is repeated, using nine rabbits. By this somewhat artificial method the final value arrived at does not deviate greatly from the true one, as is evidenced by the fact that it can be reduplicated within at least 10 per cent. of an error. This is not a serious variability, for clinical purposes, and the assay of insulin is at least as close as that of any other glandular product, with the possible exceptions of pituitrin, epinephrin, and thyroxin. It is always advisable to conduct parallel observations with a standardised preparation of insulin, and for this purpose there can be no doubt that a dried one should be used. Enough of this should obviously be kept in stock to last over a long period of time, and when a new standard must be provided its use should overlap that of the old one. Occasionally, this standard should be compared with the international one. By comparison with standards the risk of error due to seasonal variations in the susceptibility of animals towards insulin is minimised.

To illustrate the types of results obtained on different dates on the same preparation of insulin hydrochloride, the following summary of results are given (detailed protocols will be found in the report of the standardisation committee of the Health Section of the League of Nations).

Date.	Dose per 2 Kg. c.c.	Units.	Average of Each Group.	Final Average Assay.
April 14	0·075	29·6	—	—
	—	29·4	29·5	—
,,	0·100	22·0	—	—
	—	22·1	—	—
	—	30·4*	24·8	—
,,	0·125	22·2	—	—
	—	17·7	—	—
	—	20·5	20·1	24·8
April 17	0·075	28·6	—	—
,,	—	19·0	—	—
	—	21·6	23·1	—
,,	0·100	17·8	—	—
	—	13·6	—	—
	—	21·0	17·5	—
,,	0·125	16·9	—	—
	—	19·5	18·2	19·6
May 1	0·75	18·6	—	—
	—	22·3	—	—
	—	23·5	—	—
	—	25·0	—	—
	—	25·6	23	—
,,	0·100	14·6	—	—
	—	20·4	—	—
	—	15·8	—	—
	—	19·6	—	—
	—	16·6	—	—
	—	14·0	20·2	—
,,	0·125	13·6	—	—
	—	17·6	—	—
	—	13·0	—	—
	—	15·4	—	—
	—	20·4	—	—
	—	15·8	19·2	20·8
			Grand average	21·7 units.

In 1 c.c. of a solution containing 2·5 mgs. of insulin hydrochloride, 1 mg., therefore, contains 8·4 units.

Other assays of this material by the same method were as follows :—

Toronto—

April 6 and 7 . . . . . 7·9 units.
,, 8, 9, 13, 14, 15, 16, 17, and May 1 . . 8·2 ,,

Other Laboratories—

Lilly Co. . . . . 8·2-8·6 c.c.
Squibb Co. . . . . 8·45 c.c.

2. Comparative Method as Used in the National Research Laboratories.—

The rabbits, prepared as above described, are divided into two groups, A and B. Each rabbit of group A is injected with 0·5 units of the standard per kilo. body weight, and each rabbit of group B with what is believed to be an equivalent amount of the insulin to be tested.

The value of a in the above equation is then determined. At an interval of several days the observations are repeated, with the difference that the rabbits of group A now receive the unknown insulin, and those of B the standard. The unit value is then calculated from the relationship found between the effects produced by the standard and the unknown.

That this procedure cannot entirely eliminate the factor of varying susceptibility will be evident from the following results :—

Ten rabbits, each weighing to start with 2 kg. were kept during six weeks under strictly similar conditions, and each was injected once a week with the same dose, per kg. body weight, of the same preparation of insulin, although different rabbits might receive different doses.

The values of a in mg. per cent. for each rabbit on the different weeks were as follows (Macleod and Orr) :—

Rabbit.	Week.							Average.
	1	2	3	4	5	6	7	
1	38	46	25	(72)*	31	46	—	37
2	36	34	26	36	27	39	—	33
3	53	46	30	49	62	45	—	47
4	—	—	43	51	56	46	—	49
5	43	28	39	25	21	36	25	31
6	23	30	(51)	28	28	31	—	28
7	29	29	(54)	48	38	41	—	37
8	25	28	(43)	39	32	40	50	35·5
9	17	(57)	40	60	35	—	—	31
10	28	23	(63)	38	48	34	—	34

* The figures in brackets are not used in calculating the averages.

Grevenstuk and Laqueur have likewise observed that the same animal does not react equally to insulin on different occasions.

The principle of comparison with a standard which is so well carried out in this procedure does, however, eliminate errors due to variability affecting the animals, as a group, such as those due to changes in temperature conditions, unavoidable changes in diet, and other unknown factors. Now that an international standard is set up, the principle of comparison, by whatever manner it is applied, must greatly simplify the assay of insulin.

3. The Method Used in the University of Amsterdam.—

This method is based on the behaviour of the blood sugar, but consideration is also taken of the incidence of convulsions. In two and in four hours after the injection of varying doses of insulin into (20) rabbits, the blood sugar is determined and the number of animals showing convulsions is noted. The convulsive level (Krampf-grenze) is considered to be reached if the blood sugar reaches 0·045 per cent. or the animal shows convulsions. If this is reached in 75 per cent. of

the injected animals it is considered that the corresponding amount of insulin equals 3 units (Grevenstuk and Laqueur).

Certain laboratories depend exclusively on *the insidence of the symptoms of hypoglycæmia* in making the assays, and when this is done by the comparative method it is quite satisfactory. The expense of using such a method with rabbits led Fraser and Krogh to investigate the possibility of using mice for this purpose, and Krogh considers as one mouse unit of insulin that amount which causes definite symptoms in 50 per cent. of the injected animals within two hours, the animal being kept after injection at a temperature of not less than 30° C. The assay depends on comparison with a standard preparation which is injected simultaneously with the unknown into an equal number of mice from a common stock.

In our experience this method, even when it is carried out with every precaution as to the condition of the animals, etc., is not so dependable as that based on the lowering of blood sugar, and in any case there are several theoretical objections to the convulsive method, although when used by the comparative principle it is undoubtedly a most useful one. The chief of the objections are as follows :—

(1) Although the symptoms usually occur in recently fed rabbits when the blood sugar reaches 0·045 per cent., they may not do so in starved animals until a much lower level is reached (McCormick, Macleod and Noble, Grevenstuk and Laqueur, Clough, Allen and Root, etc.).

(2) The tendency for symptoms to appear when the blood sugar stands at 0·045 per cent. is said to have diminished as purer preparations have become available. This has been particularly emphasized by Grevenstuk and Laqueur, who have compared the proportion of rabbits showing symptoms at the convulsive level with those not showing them, for ten consecutive periods extending over eighteen months. Ninety-six per cent. of the animals showed convulsions in the first period, but the proportion steadily decreased in each successive period, until in the last one only 41 per cent. showed convulsions. This would seem to indicate that the lowering of blood sugar, *per se*, cannot be the sole cause of the symptoms. Grevenstuk and Laqueur suggest that they are really due to admixture of the insulin with some substance which causes them after the blood sugar has been reduced. It may, however, be that the purer preparations act more quickly and thus cause the blood sugar

to descend at a rate with which the tension in the tissues does not keep pace (see p. 277).

(3) Sometimes convulsions or symptoms simulating them appear before the blood sugar has reached the usual convulsive level (Macleod and Orr, Grevenstuk and Laqueur).

(4) The method at best only indicates whether the blood sugar has reached the possible convulsive level, and it gives no information with regard to the duration of the hypoglycæmic effect.

(5) For use in the treatment of diabetes mellitus, it is not the tendency of the preparation to produce convulsions that is of importance, but its influence on the blood sugar.

Other Methods of Assay.—Methods of assay based on other principles than the lowering of blood sugar or the incidence of hypoglycæmic symptoms have been tried from time to time. Several of these have been referred to incidentally in other chapters and will require little further notice in this one :—

(1) Since the curve expressing the relationship between the units of insulin injected and the degree of lowering of blood sugar shows that the greatest differences in the latter are obtained when small doses are used, it was considered possible that an extension of this part of the curve might be obtained by increasing the amount of blood sugar on which the insulin could act. This was done either (exogenously) by the injection of glucose solutions (see p. 207), or (endogenously) by the use of epinephrin (Eadie and Macleod). In actually applying the former method 2 gms. of glucose per kilo body weight were injected subcutaneously into each of several rabbits which had received varying doses of insulin seventy-five minutes previously. It was hoped that the extent to which the blood sugar rose would then bear a simple relationship to the dose of insulin, but the results were no more satisfactory than those obtained on normal animals. In the latter method, which had previously been suggested by Zuelzer for the pancreatic extracts prepared by him (p. 57), equal quantities of a carefully standardised preparation of epinephrin, and varying amounts of insulin, were injected into well-fed rabbits, and although an interesting relationship was found to exist (see p. 230), the method did not prove to be of practical service (Eadie and Macleod).

(2) There can be little doubt that the variability of different animals towards insulin, after every precaution has been taken

with regard to diet, environment, etc., is due to the influence exercised on carbohydrate metabolism through the internal secretion of insulin from the pancreas and of hormones, such as thyroxin (p. 337), and possibly of epinephrin (p. 232) and pituitrin (p. 340) from other ductless glands. It has been attempted to eliminate these influences. Thus F. N. Allan has used depancreatised animals, and although he has shown that a very close relationship exists between the dose of insulin and the amount of glucose metabolised by the animal—glucose equivalents (p. 96)—the method did not prove itself to be one of practical value for purposes of assay.

Bodansky, Burn, and Dale have used thyroidectomised animals (rabbits), and the latter have reported a decidedly greater constancy in the assays of insulin carried out on them, as compared with those on normal animals. Stewart and Rogoff have used rabbits adrenalectomised by their method without finding that this involves any difference in the response to insulin. Cannon, McIver, and others, on the contrary, found that the rate of recovery of blood sugar is influenced after adrenalectomy.

It is at least safe to conclude that none of these methods is sufficiently superior to that depending on the use of the normal rabbit to warrant their employment for purposes of assay. Various attempts have been made to discover some test tube reaction of insulin that could be used. Thus, the bleaching of methylene blue, *in vacuo*, in the presence of fresh tissue and glucose (Thunberg's method), has been tried (p. 320), and also the similar effect produced by yeast, but with no success.

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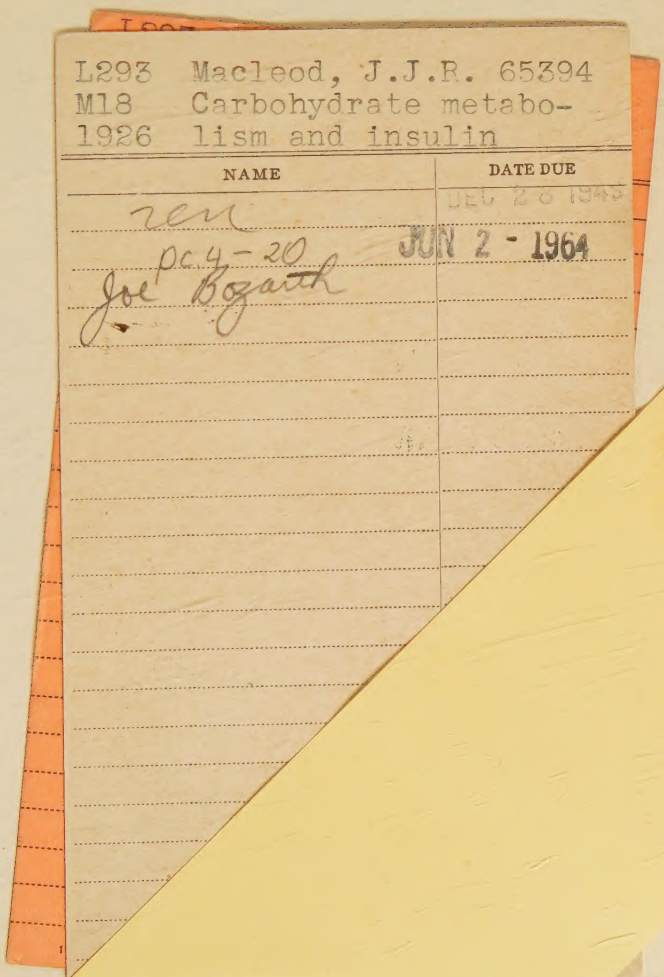
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